The Intergenerational Consequences of Fetal Programming

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Abstract

Numerous epidemiological studies in diverse populations have demonstrated a link between low birth weight and a number of cardiovascular risk factors. This association has given rise to the 'fetal programming hypothesis', which proposes that a stimulus or insult acting during critical periods of growth and development may permanently alter tissue structure and function, leading to later disease.

There is increasing evidence that this phenomenon may not be limited to the first generation offspring; both human and animal studies have demonstrated intergenerational effects on birth weight and cardiovascular risk; however the mechanisms behind this remain unclear.

One proposed mechanism to explain the early life origins of disease is fetal overexposure to glucocorticoids. We have explored the intergenerational effects in the dexamethasone-programmed rat, a model of fetal programming in which *in utero* exposure to excess glucocorticoid results in low birth weight offspring, which develop glucose intolerance in adulthood, thought to be secondary to an increase in the activity of a key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK). Using this model we have demonstrated programming effects in a second generation of animals, which also showed reduced birth weight, elevated PEPCK and glucose intolerance; effects which had resolved by the third generation. Although the persistence of such programming effects in subsequent generations may be secondary to programmed alterations in maternal physiology, resulting in an adverse environment for the developing fetus, we also demonstrate a clear effect of paternal phenotype on offspring birth weight and PEPCK, suggesting that the father also has an effect on the intergenerational transfer of disease risk.

The association between low birth weight and later disease is amplified by the development of obesity in humans and in animal models of maternal undernutrition. We have developed a model of high fat feeding in Wistar rats and used this to explore the effect of obesity in the dexamethasone-programmed rat. After 20 weeks on a high fat diet, there were no differences in body weight and insulin resistance between rats exposed to dexamethasone *in utero* and control animals; however there was a marked increase in hepatic triglyceride content in the programmed group.

Thus, the amplification of the programming effect by obesity may be related to changes in hepatic metabolism and insulin resistance, which may be glucocorticoid dependent.

These observations demonstrate intergenerational effects of fetal programming by glucocorticoids, which cannot be explained entirely by the exposure of the fetus to a programmed adverse maternal environment and indicate the potential importance of epigenetic factors in the intergenerational inheritance of the 'programming effect'. In addition we have discovered a potential mechanistic link by which the postnatal environment may differentially affect programmed animals.

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Declaration

I declare that this thesis was written by me and that the data presented within it is a result of my own work, except where specifically acknowledged in the text.

I declare that this work has not been submitted for any other degree.

Amanda J Drake, Edinburgh, January 2004

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Abbreviations

A	11-dehydrocorticosterone
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
B	Corticosterone
BP	Blood pressure
BMI	Body mass index
BSA	Bovine serum albumin
C	Control diet
CBG	Corticosteroid-binding globulin
CRH	Corticotrophin-releasing hormone
CTP	Cytosine triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA

DNA	Deoxyribonucleic acid
Dex	Dexamethasone
DC	F1 dex animals fed a control diet
DHF	F1 dex animals fed a high fat diet
DTT	Dithiothreitol
DEPC	Diethypyrocarbonate
E	Cortisone
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
E0	Gestational day 0
F	Cortisol
F1	First generation
F2	Second generation
F3	Third generation
FFA	Non-esterified fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GR	Glucocorticoid receptor
HF	High fat diet
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
HPA	Hypothalamic-pituitary adrenal axis
IGF	Insulin-like growth factor
IGF2R	Insulin-like growth factor 2 receptor
INS-VNTR	Variable number tandem repeat minisatellite 5' to insulin gene
LDL	Low-density lipoprotein
mRNA	Messenger RNA
MR	Mineralocorticoid receptor
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PPAR	Peroxisome-proliferator activated receptor

PEPCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
SEM	Standard error of the mean
sTAR	Steroid acute regulatory protein
RNA	Ribonucleic acid
RT	Reverse transcription
TG	Triglycerides
TLC	Thin layer chromatography
TNFα	Tumour necrosis factor α
Veh	Vehicle
VC	Vehicle animals fed a control diet
VHF	Vehicle animals fed a high fat diet

Publications from this thesis

Full papers

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Drake AJ, Reidy L, Livingstone DEW, Andrew R, Seckl JR, Walker BR. Contrasting changes in glucocorticoid metabolism during high fat feeding in rats. Manuscript in preparation.

Drake AJ, Walker BR, Seckl JR. The intergenerational effects of prenatal glucocorticoid programming. Manuscript in preparation

Abstracts

Drake AJ, Cleasby ME, Livingstone DEW, Seckl JR, Walker BR. Reduced glucocorticoid receptor expression in obese Zucker rats: protection from the metabolic consequences of obesity? Presented at the British Endocrine Societies meeting, April 2002.

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Chapter One – Introduction

Many epidemiological studies in distinct populations in the UK and the rest of the world have demonstrated an association between low birth weight and the subsequent development of hypertension, insulin resistance, type 2 diabetes and cardiovascular disease (Barker 1998). 'Fetal programming' has been proposed as the mechanism underlying this association between low birth weight, childhood growth and subsequent disease. The fetal programming hypothesis proposes that a stimulus or insult acting during critical periods of growth and development may permanently alter tissue structure and function. Indeed, evidence from both human and animal studies (Langley-Evans et al 1996c, Barker 1998, Nyirenda et al 1998, Doyle et al 2000) suggests that many diseases of adult life can be induced by manipulating the environment of the fetus. There is also evidence that this phenomenon may not be confined to the first generation; intergenerational effects on birth weight, blood pressure and glucose tolerance have been reported in both human and animal studies (Stewart et al 1975, Alcolado & Alcolado 1991, Emanuel et al 1992, Davey Smith et al 1997, Hoet & Hanson 1999), although the mechanisms remain unclear. We and others have provided evidence that programming effects may be mediated by glucocorticoids (Benediktsson et al 1993, Nyirenda et al 1998, Gatford et al 2000, Newnham 2001, Sloboda et al 2002b) and have developed an animal model of glucocorticoid programming (Benediktsson et al 1993). The first half of this thesis explores the intergenerational effects of programming in this model, the dexamethasone-programmed rat.

Glucocorticoid secretion and metabolism is altered in obesity in humans and animal models (Andrew et al 1998, Livingstone et al 2000a, Rask et al 2001, Pasquali et al 2002, Lindsay et al 2003, Wake et al 2003). Obesity appears to be important in modifying the risk of later disease associated with low birth weight (Phillips et al 1994) and amplifies programming effects in some animal models (Vickers et al 2000). In the dexamethasone-programmed rat, local alterations in glucocorticoid signalling may mediate the observed metabolic changes (Nyirenda et al 1998, Cleasby et al 2003a); the programmed animal may therefore be more susceptible to

obesity and its sequelae. The second half of this thesis explores the amplification of the programming phenotype by obesity.

This introductory chapter reviews the evidence and potential mechanisms for fetal programming and for intergenerational effects in human and animal models. The role of obesity in the amplification of programming is also discussed. Glucocorticoid physiology and metabolism is reviewed, with particular emphasis on the role of glucocorticoids in obesity. Finally, the aims of this thesis are listed.

1.1 Background

The association between low birth weight and later disease has now been described in many populations (Barker 1998) and appears to be independent of classical lifestyle risk factors such as smoking, adult weight, social class, alcohol and lack of exercise, which are additive to the effect (Barker et al 1993a). Importantly, the association holds for the full range of birth weights, including those within the normal range. In addition, a number of studies have suggested that faster post-natal catch-up growth may also be predictive of later risk of cardiovascular disease (Barker et al 1993a, Bavdekar et al 1999, Eriksson et al 1999, Forsen et al 2000, Law et al 2002).

The relative importance of genetic and environmental factors in this phenomenon remains unknown. Two major environmental hypotheses have been proposed to explain the mechanism by which low birth weight is associated with adult disease: fetal undernutrition (Barker & Osmond 1986); and overexposure of the fetus to glucocorticoids (Edwards et al 1993). A third hypothesis suggests that genetic factors may lead to both low birth weight and subsequent risk of cardiovascular disease; indeed, genetic loci have recently been described which may link smallness at birth with adult disease (Dunger et al 1998, Hattersley et al 1998) (Figure 1.1).

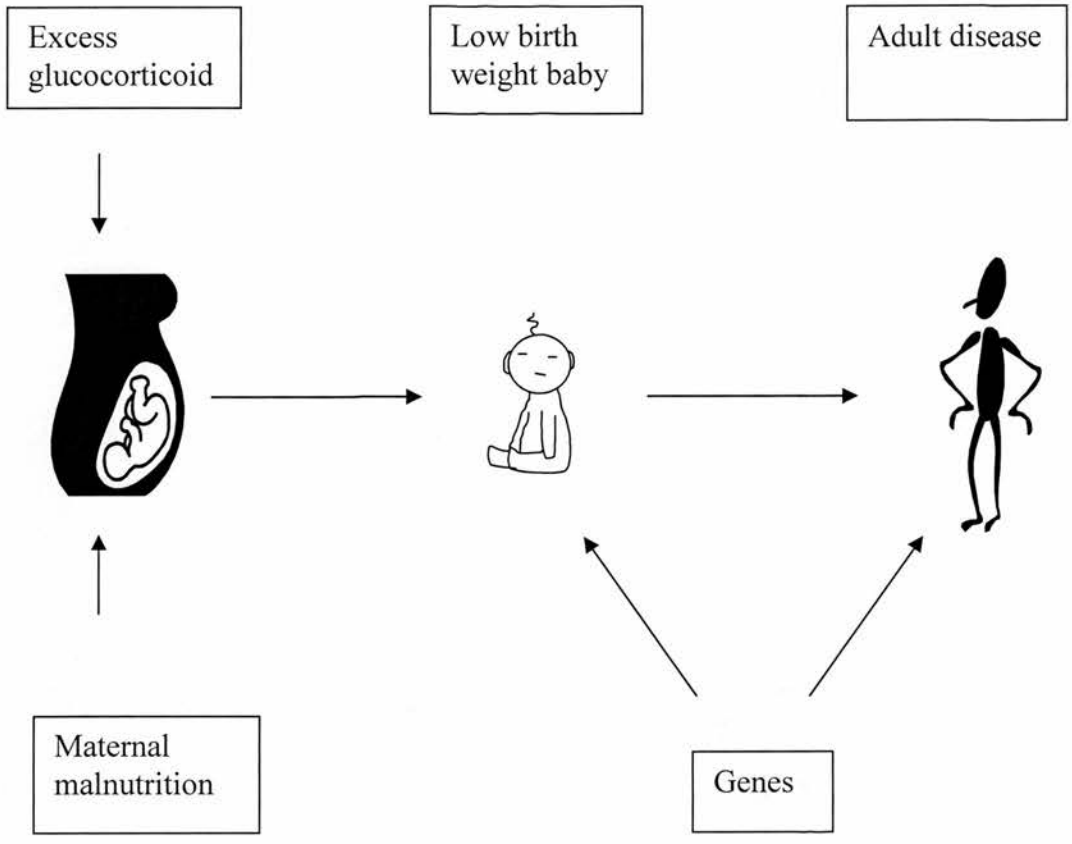


Figure 1.1 Potential mechanisms for fetal programming.

Two major environmental hypotheses have been proposed to explain the mechanism by which low birth weight is associated with adult disease: fetal undernutrition; and overexposure of the fetus to glucocorticoids. A third hypothesis suggests that genetic factors may lead to both low birth weight and subsequent risk of cardiovascular disease, for example a genetically mediated insulin resistance may lead to both reduced insulin-mediated fetal growth, and later disease.

1.2 Fetal programming

Many reports have now described links between low birth weight, fetal environment and the risk of later disease in humans (Barker 2002). Epidemiological studies have demonstrated associations between fetal and infant growth and the risk of developing impaired glucose tolerance, type 2 diabetes, coronary heart disease, hypertension and hyperlipidaemia; a cluster of factors known as the metabolic syndrome (reviewed in Barker 1998, Barker 2002).

1.2.1 Cardiovascular disease

Initial clues that fetal development may be associated with later disease came from epidemiological studies demonstrating that death rates from cardiovascular disease were highest in areas of the UK that had the highest infant mortality during the early 20^{th} century (Barker & Osmond 1986). The Hertfordshire cohort comprised 16,000 men and women born in Hertfordshire between 1911 and 1930, and in these individuals, death rates from coronary heart disease decreased with increasing birth weight (Osmond et al 1993). Similar findings have been reported from other cohorts in the UK (Barker et al 1993c) and elsewhere in the world (Rich-Edwards et al 1997, Forsen et al 1999, Eriksson et al 2000). Many studies have also shown an association between low birth weight and hypertension (reviewed in Barker 1998).

1.2.2 Type 2 diabetes

Studies in the Hertfordshire and Preston cohorts have shown that low birth weight and thinness at birth is associated with insulin resistance (Barker et al 1993b, Phipps et al 1993, Phillips et al 1994). These observations have been confirmed in Sweden (Lithell et al 1996), Finland (Forsen et al 2000, Eriksson et al 2002) and the US (Rich-Edwards et al 1999).

1.2.3 The hypothalamic-pituitary adrenal (HPA) axis

Low birth weight is also associated with long-term effects on the HPA axis (Phillips et al 1998, Phillips et al 2000). Individuals with lower birth weight have evidence of chronic activation of the HPA axis (Levitt et al 2000, Reynolds et al 2001). As increased cortisol concentrations in blood, saliva and urine are associated with features of the metabolic syndrome (Phillips et al 1998, Phillips et al 2000), programming of the HPA axis may underlie the association between low birth weight and later disease.

1.2.4 Animal models of fetal programming

Potential mechanisms underlying fetal programming have been explored using a number of animal models. Both maternal undernutrition (low protein or low calorie diets during gestation) and *in utero* overexposure to glucocorticoids results in offspring of lower birth weight which subsequently develop glucose intolerance, hypertension and HPA axis activation (reviewed in Ozanne 2001). These models will be discussed in more detail below.

1.3 Potential mechanisms for fetal programming

1.3.1 Glucocorticoid programming

Steroid hormones are typically associated with long-term organisational effects and are therefore prime candidates to explain programming. For example, neonatal exposure to androgens permanently programmes the expression of hepatic steroid metabolising enzymes and the development of sexually dimorphic structures in the anterior hypothalamus as well as sexual behaviour (Arai & Gorski 1968, Gustafsson et al 1983). These effects can only be exerted during a specific perinatal period, and the mechanisms may reflect the influence of androgens on organ growth and maturation. In the rat, the sexually dimorphic nucleus of the hypothalamic preoptic

area is larger in males. Testosterone inhibits apoptosis specifically between postnatal days 6 and 10 in this nucleus, producing the male phenotype (Davis et al 1996).

Fetal glucocorticoid overexposure has been proposed as a mechanism underpinning the early life programming of adult disease. Glucocorticoid receptors (GR) are expressed in most fetal tissues from early in gestation (Cole et al 1995) and are also expressed in the placenta (Sun et al 1997). The higher affinity mineralocorticoid receptor (MR) is also expressed in fetal tissues, but has a more limited tissue distribution and is present at a later gestational stage (Brown et al 1996b). Perinatal glucocorticoids alter the rate of maturation of various organs, such as the lung (Ward 1994), heart (Bian et al 1992, Bian et al 1993) and kidney (Celsi et al 1997) and are used therapeutically in threatened preterm labour for this reason. In addition to the widespread use of glucocorticoids to enhance fetal lung maturation in the management of women at risk of preterm delivery, they are also used in the antenatal management of fetuses at risk of congenital adrenal hyperplasia.

Glucocorticoid treatment during pregnancy reduces birth weight in animals including non-human primates (Reinisch et al 1978, Ikegami et al 1997, Nyirenda et al 1998, Newnham et al 1999, Newnham & Moss 2001). Human studies have confirmed that antenatal glucocorticoids are associated with a reduction in birth weight (French et al 1999, Bloom et al 2001), although normal birth weight has been reported in infants at risk of congenital adrenal hyperplasia receiving low dose dexamethasone *in utero* from the first trimester (Forest et al 1993, Mercado et al 1995). Fetal cortisol levels are increased in human fetuses with intrauterine growth retardation or in pregnancies complicated by pre-eclampsia, which may indicate a role for endogenous cortisol in fetal growth retardation (Goland et al 1993, Goland et al 1995). Cortisol also affects placental size, the effect dependent on the dose and the timing of exposure (Gunberg 1957).

Many glucocorticoid sensitive systems are affected by early-life programming. Glucocorticoids are essential for normal brain development, exerting a number of effects in most regions of the developing brain including the hippocampus and the hypothalamic-pituitary-adrenal (HPA) axis and perinatal glucocorticoids have been shown to programme specific effects in the brain (Welberg & Seckl 2001).

Glucocorticoids are known to increase blood pressure in adults. Cortisol elevates the blood pressure in fetal sheep when infused directly *in utero* (Tangalakis et al 1992) and at birth in humans (Kari et al 1994) and in sheep (Berry et al 1997). The administration of betamethasone to pregnant baboons elevates fetal blood pressure (Koenen et al 2002). Antenatal glucocorticoid exposure also leads to permanently elevated blood pressure in later life. Rats treated with dexamethasone *in utero* have elevated blood pressure in adulthood (Benediktsson et al 1993, Sugden et al 2001), as do sheep exposed to excess glucocorticoid *in utero* either as maternally-administered dexamethasone or as a maternal cortisol infusion (Dodic et al 2001, Dodic et al 2002a, Dodic et al 2002b, Jensen et al 2002). The timing of glucocorticoid exposure appears to be important; exposure to glucocorticoids during the final week of pregnancy is sufficient to produce permanent adult hypertension in the rat (Levitt et al 1996), whereas in sheep, such effects are seen after glucocorticoid exposure earlier in gestation (Gatford et al 2000).

Maternal glucocorticoid administration has an effect on cord glucose and insulin levels in the ovine fetus (Sloboda et al 2002b) and on glucose homeostasis in the adult offspring. Intriguingly, in sheep, antenatal glucocorticoid exposure with or without fetal growth restriction altered glucose metabolism (Moss et al 2001). Maternal but not fetal injections of betamethasone restricted fetal growth (Newnham et al 1999), although offspring of both groups had altered glucose metabolism postnatally (Moss et al 2001). In rats, last trimester glucocorticoid exposure programmes permanent hyperglycaemia and hyperinsulinaemia in the adult offspring (Nyirenda et al 1998). Again, the timing of exposure is important; earlier treatment does not result in adult glucose intolerance.

1.3.2 Nutritional programming

Animal studies have shown that maternal undernutrition during pregnancy results in reduced birth weight and a number of metabolic and cardiovascular sequelae. Rats maintained on a low protein diet throughout pregnancy deliver offspring with reduced birth weight, which develop glucose intolerance and hypertension in adulthood (Langley et al 1994, Langley & Jackson 1994, Hales et al 1996). Other

studies have demonstrated permanent effects of maternal protein restriction on the endocrine pancreas (Snoeck et al 1990), the liver (Ozanne et al 1996a), and skeletal muscle (Ozanne et al 1996b).

The role of undernutrition in fetal programming in humans is less clear. The effects of malnutrition have been reported widely following the Dutch famine of 1944 – 1945, during which the Western Netherlands was affected by acute famine, a period known as the 'Hunger Winter'. The famine ceased immediately with liberation in May 1945, after which food supplies became abundant. Babies exposed to famine during the last trimester were of lower birth weight and developed glucose intolerance in adulthood (Ravelli et al 1998). However, not all studies show a strong association between maternal undernutrition and later offspring disease. The siege of Leningrad, which lasted from 1941 until 1944, led to a severe famine during which up to one million people died, mainly of starvation. Average male and female birth weights fell by up to 18% during this time. Despite this, individuals exposed to famine *in utero* showed no difference in the prevalence of glucose intolerance, dyslipidaemia, hypertension or cardiovascular disease in adulthood, when compared with a similar group exposed to the same famine conditions in infancy, and a group not exposed to famine (Stanner et al 1997). However, there are differences between the Dutch and Leningrad famine survivors that may explain the differences found. One of the major hypotheses advanced to explain this discrepancy suggests that the lack of catch-up growth in children in the Leningrad population, which was malnourished before and after the severe famine, may explain this apparent protection from the programming effects seen in the Dutch cohort.

In developed countries, dietary deficiency is rarely thought to be a significant cause of impaired fetal growth (Godfrey & Robinson 1998, Mathews et al 1999, Robinson et al 2000). However, recent studies suggest that women with eating disorders are more likely to give birth to low birth weight infants (Conti et al 1998). Although dietary supplementation has been shown to increase birth weight in a study of women in the Gambia (Ceesay et al 1997), most studies have only demonstrated a small effect (Kramer & Kakuma 2003). Intriguingly, one small study in Guatemala has demonstrated that nutritional supplementation of girls during early childhood

may have a positive effect on the birth weight and height of their offspring (Stein et al 2003).

Birth weight is influenced by maternal body composition as well as nutrition during pregnancy (Barker 1998) and it is likely that fetal development may be influenced by micronutrient as well as macronutrient deficiency (Boucher 1998). However, so far, single micronutrient supplementation trials have generally not shown a significant effect on birth weight (Fall et al 2003, Merialdi et al 2003).

1.3.3 A common mechanism?

Intriguingly, it is possible that glucocorticoid programming and maternal undernutrition are linked by a common mechanism (Langley-Evans et al 1996c). The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD 2) converts active glucocorticoids to inactive products, is present in the placenta, and may act as a barrier to protect the fetus from maternal glucocorticoids (Edwards et al 1993). In rats, inhibition of this enzyme by carbenoxolone results in offspring of low birth weight, with later glucose intolerance and high blood pressure (Lindsay et al 1996a, Lindsay et al 1996b), a phenotype similar to that seen in rats exposed to dexamethasone in utero (Benediktsson et al 1993, Nyirenda et al 1998). Reduced placental 11β-HSD 2 gene expression has been reported in human pregnancies complicated by intra-uterine growth retardation (McTernan et al 2001), and rodent studies have demonstrated that dietary restriction during pregnancy reduces placental 11β-HSD 2 expression and disturbs the neonatal HPA axis (Langley-Evans et al 1996c, Lesage et al 2001). Thus, maternal malnutrition potentially exerts programming effects by inducing fetal overexposure to the effects of maternal glucocorticoids.

1.4 Intergenerational programming

1.4.1 Intergenerational programming in humans

There is evidence that what appears to be inherited, and may therefore be assumed to be due to genetic factors, may in fact, be due to a perpetuation of programming effects across a number of generations. There is a well-recognised 'intergenerational cycle of growth failure' in the developing world – young girls who grow poorly become short women and are more likely to give birth to low birth weight babies. If these infants are girls, they are likely to continue the cycle by being short in adulthood and so on (Ramakrishnan et al 1999). It is possible that this apparent intergenerational effect on fetal growth may also be important in the developed world, and may contribute to adult cardiovascular disease in Westernised societies.

In 1986, Emanuel defined intergenerational influences as 'those factors, conditions, exposures and environments experienced by one generation that relate to the health, growth and development of the next generation' (Emanuel 1986). Epidemiological studies have suggested that there may be intergenerational effects on birth weight (Ounsted & Ounsted 1968, Johnstone & Inglis 1974, Klebanoff et al 1984, Emanuel et al 1992, Hennessy & Alberman 1998, Collins et al 2002). If low birth weight is associated with increased cardiovascular risk, this could lead to the 'inheritance' of a predisposition to low birth weight and adverse cardiovascular risk across a number of generations. A model is presented in figure 1.2.

Potential explanations for intergenerational effects include: a) that genetic attributes may manifest themselves similarly in parent and offspring; b) that adverse extrinsic environmental conditions may persist across generations; and c) that adverse *in utero* experiences may permanently affect maternal growth and development, altering her metabolism in such a way as to provide an adverse environment for her fetus. This last hypothesis suggests a mechanism by which programming effects could be self-perpetuating through several generations.

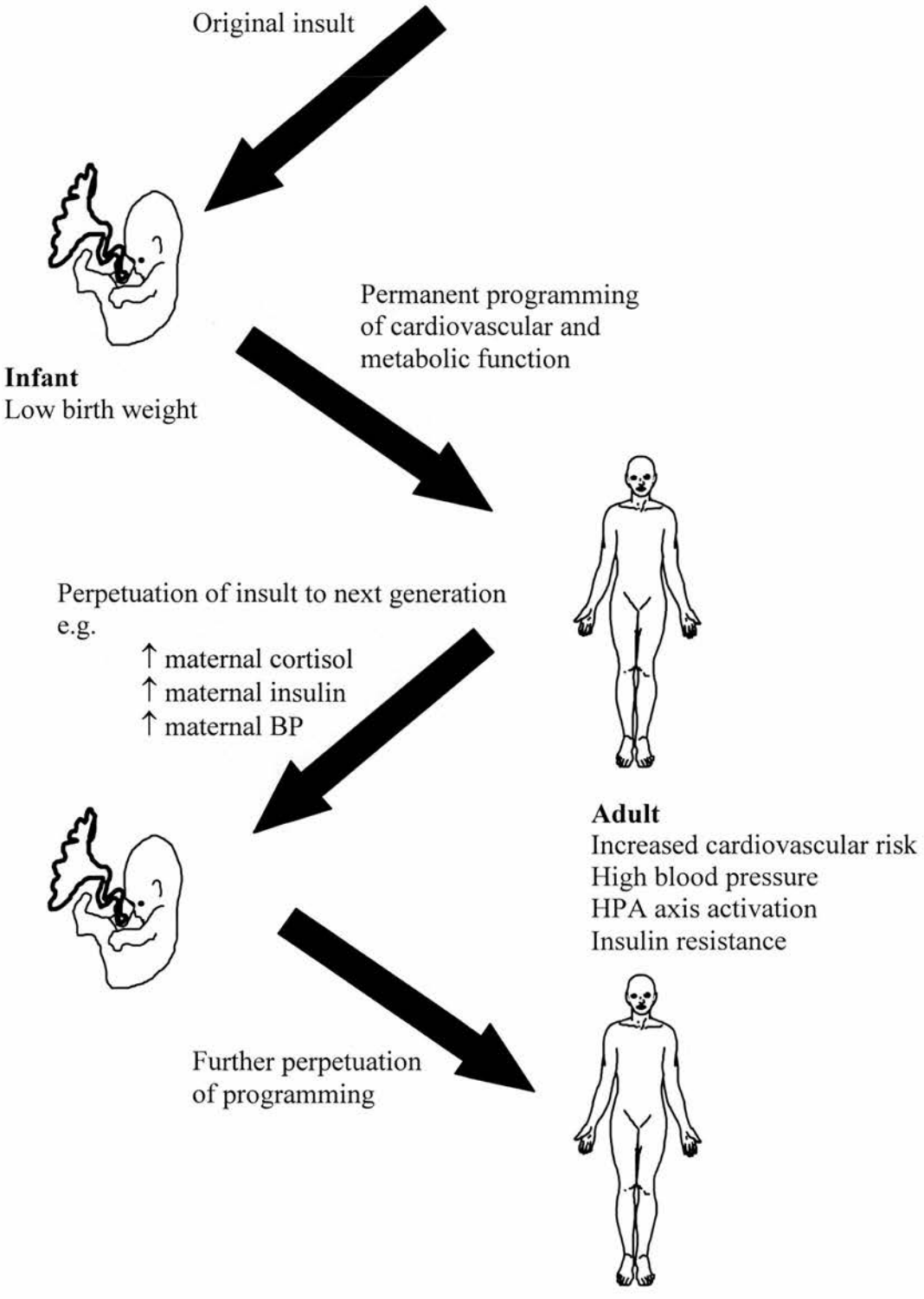


Figure 1.2 Proposed model for intergenerational programming of birth weight and cardiovascular risk.

HPA: Hypothalamic-pituitary-adrenal axis

1.4.2 Intergenerational effects on birth weight

Studies in a number of different populations have demonstrated that offspring birth weight is related to maternal birth weight (Ounsted & Ounsted 1968, Johnstone & Inglis 1974, Klebanoff et al 1984, Carr-Hill et al 1987, Klebanoff et al 1989, Emanuel et al 1992, Klebanoff et al 1997, Emanuel et al 1999, Collins et al 2002), an effect which appears to act through reduced intrauterine growth rather than reduced duration of gestation (Klebanoff et al 1984, Klebanoff et al 1997). In 1968, Ounsted published data from a cohort of growth retarded and growth-accelerated infants taken from the British Perinatal Mortality survey of 1958 suggesting a matrilineal pattern of birth weight inheritance (Ounsted & Ounsted 1968) although the study was too small to exclude a major contribution of the father to offspring birth weight. Subsequently, extensive study of the 1958 birth cohort from the British National Child Development Study showed a direct association between parental and offspring birth weights (Alberman et al 1992, Emanuel et al 1992, Hennessy & Alberman 1998), and assessment of grandparental data in these and other studies has also provided evidence for a matrilineal multigenerational effect on birth weight (Emanuel et al 1992, Klebanoff et al 1997). Although there is also a significant relationship between paternal and offspring birth weight (Emanuel et al 1992, Klebanoff et al 1998, Magnus et al 2001), this association is not as strong as that for maternal birth weight (Alberman et al 1992, Emanuel et al 1992, Coutinho et al 1997, Klebanoff et al 1998).

Intergenerational effects on birth weight are also seen in the developing world. Prospective studies in rural Guatemala found a clear relationship between maternal and offspring birth weight, with an effect nearly twice that seen in developed countries (Ramakrishnan et al 1999).

In addition, secular trends in birth weight may be mediated by intergenerational factors. Studies in the US have identified differing intergenerational birth weight effects among African-American and white populations in Illinois depending on place of birth (Collins et al 2002), suggesting that environmental factors may be influencing the secular trends in birth weight in populations with the same racial background.

1.4.3 Intergenerational effects on cardiovascular risk factors

The link between low birth weight and adult cardiovascular risk could be confounded if both low birth weight and cardiovascular risk were inherited together. If this were the case, then we might expect both parents to have an equal influence on the inheritance of birth weight and later cardiovascular disease. However, there is much evidence that parental influence is not equal and that the association of maternal cardiovascular risk with offspring birth weight and cardiovascular risk is stronger than the paternal association. A number of recent studies have explored the relationship between parental cardiovascular risk and offspring characteristics and several have demonstrated an association between offspring birth weight and parental cardiovascular risk (Davey Smith et al 1997, Davey Smith et al 2000a, Davey Smith et al 2000b, Smith et al 2001, Lawlor et al 2003a, Lawlor et al 2003b). Whilst a further study showed no difference between maternal and paternal coronary heart disease transmission to male offspring (Kinra et al 2003), others have shown a stronger association with maternal cardiovascular disease (Davey Smith et al 1997, Kuznetsova et al 2003).

There is a well-documented relationship between parental and offspring blood pressure (Watt et al 1991, Walker et al 1998). There is also evidence that the inheritance of low birth weight and hypertension may be linked. Mothers with higher blood pressure in later life have been shown to deliver smaller babies, a relationship that confounds that which is found between low birth weight and adult hypertension (Walker et al 1998, Lawlor et al 2002). Women who were small for gestational age are at increased risk of developing hypertension during pregnancy (Klebanoff et al 1999), and a number of studies have found that women with hypertension during pregnancy are at increased risk of delivering low birth weight babies (Misra 1996, Ferrer et al 2000, Brown et al 2001, Buchbinder et al 2002). Additionally, there is a continuous inverse association between fetal growth and maternal blood pressure across the range seen in normal pregnancy; higher maternal diastolic blood pressure during later pregnancy is predictive of lower offspring birth weight (Churchill et al 1997). However, although paternal blood pressure is associated with offspring blood pressure, it does not predict offspring birth weight (Walker et al 1998). Furthermore,

lower maternal but not paternal birth weight is related to higher offspring blood pressure, an association which is largely independent of the relationship between maternal and offspring birth weight (Barker et al 2000).

There have been many studies of the association of parental diabetes with offspring diabetes risk (Alcolado & Alcolado 1991, Thomas et al 1994, Klein et al 1996, Viswanathan et al 1996, Karter et al 1999, Dabelea et al 2000, Lindsay et al 2000, Meigs et al 2000, Yajnik et al 2001). Studies of women in the US Nurses study, and those from Pima Native American populations have demonstrated that the offspring of mothers with diabetes during pregnancy were at increased risk of developing type 2 diabetes (Pettitt et al 1993, Dabelea et al 2000, Lindsay et al 2000). A further study involving 15,000 families showed a modest excess maternal transmission of type 2 diabetes in a multiethnic population in the US, although the relationships varied between racial groups and appeared stronger in the female offspring (Karter et al 1999). A number of other studies have also reported excess maternal transmission of type 2 diabetes (Alcolado & Alcolado 1991, Lin et al 1994, Thomas et al 1994, Groop et al 1996, Klein et al 1996, Riley et al 1997, Bjornholt et al 2000), although this has not been a consistent finding in all populations studied (Mitchell et al 1995, McCarthy et al 1996, Viswanathan et al 1996, Meigs et al 2000).

The association between maternal diabetes, offspring birth weight and subsequent diabetes is complex. Women of low birth weight are at increased risk of developing gestational diabetes and type 2 diabetes (Williams et al 1999, Egeland et al 2000, Forsen et al 2000, Innes et al 2002, Seghieri et al 2002). The offspring of women with gestational and type 2 diabetes are more likely to be of higher birth weight (Silverman et al 1991) and indeed, women of low birth weight who develop gestational diabetes are at increased risk of delivering a macrosomic baby (Seghieri et al 2002). Such offspring are themselves at risk of developing obesity (Pettitt et al 1993, Gillman et al 2003), gestational diabetes (Innes et al 2002) and type 2 diabetes later in life (Pettitt et al 1993, Lindsay et al 2000, Catalano et al 2003). Consistent with this, some studies have confirmed a U-shaped relationship between a woman's risk for developing gestational diabetes and her own birth weight (Egeland et al 2000, Innes et al 2002).

The Pima Indian Community has a high prevalence of maternal diabetes. In this population, low birth weight is associated with the subsequent development of type 2 diabetes, but only if paternal diabetes is also present (Lindsay et al 2000). Maternal diabetes is associated with an increased risk of type 2 diabetes in the offspring, but in individuals with higher birth weight (Pettitt et al 1993, Dabelea et al 2000, Lindsay et al 2000). A number of other studies have also shown paternal diabetes to be associated with lower offspring birth weight (Yajnik et al 2001, Hypponen et al 2003), although others have found no such association (Rich-Edwards et al 1999). In populations at high risk of type 2 diabetes it is possible that the relationship between maternal diabetes and offspring birth weight and diabetes risk is obscured by the effect of maternal metabolic disturbances on fetal growth (Pettitt et al 1993, Lindsay et al 2000), but it has also been proposed that the association of low birth weight and diabetes reflects the influence of genetic factors (Dunger et al 1998, Hattersley & Tooke 1999). Indeed, it has recently been shown that in the Pima population, birth weight shows linkage to chromosome 11, with evidence for an imprinted, paternally expressed gene (Lindsay et al 2002a). There is, however, little evidence of parent-specific linkage of diabetes to this chromosome, and indeed a number of genes associated with insulin resistance are not associated with birth weight in the Pima population (Lindsay et al 2002b).

Thus, although there are maternal and paternal effects on offspring birth weight and susceptibility to type 2 diabetes and cardiovascular disease, which may be genetic, there is evidence for a specific effect of maternal rather than paternal characteristics, which may represent a process of intergenerational programming.

1.4.4 Intergenerational effects in animal models of programming

Extensive animal studies have attempted to dissect and validate the mechanisms of the early life origins of disease (Benediktsson et al 1993, Seckl 1997, Seckl 1998, Hoet & Hanson 1999, Challis et al 2001, Meaney 2001) and there is a substantial body of evidence from these animal studies that programmed phenomena can be perpetuated in later generations. Animal models of prenatal programming by

nutrition or exercise, and postnatal programming by nutrition have shown effects on birth weight (Stewart et al 1975, Pinto & Shetty 1995) and glucose tolerance (Martin et al 2000, Patel et al 2001) in subsequent generations. Additionally, brief daily handling of rats in the neonatal period, which results in decreased stress reactivity in adulthood, is associated with intergenerational effects on the offspring hypothalamic-pituitary axis (Francis et al 1999).

1.4.4.1 Programming of birth weight

Intergenerational effects of dietary manipulations on birth weight have been demonstrated in black-and-white hooded rats (Stewart et al 1975). Colonies of rats were maintained for 12 generations on a control diet or a diet marginally deficient in protein. Birth weight was reduced in the first generation of malnourished animals and this effect of poor maternal diet on birth weight appeared to be amplified in subsequent generations. Mid-way through this intergenerational experiment a more unpalatable diet was introduced by chance. Following this, further reduction in birth weight was seen in the malnourished colony and in addition, there was a slight reduction in birth weights in the control colony. After the re-introduction of more palatable chow, birth weights in control animals increased, but did not return to baseline for approximately three generations, despite the resumption of normal nutrition. The study clearly demonstrates that continued poor maternal nutrition produces amplified effects on birth weight through a number of generations. However, the accidental introduction of less palatable food, which resulted in a period of self-imposed calorie restriction in the 'control' animals, also provides evidence that poor nutrition in one generation can produce effects on birth weight in subsequent generations. This effect was confirmed in further experiments when a number of animals from the malnourished colony weaned onto the control diet did not achieve an adult size equivalent to that of the control animals for 3 further generations (Stewart et al 1980).

Intergenerational effects on birth weight in rats have also been shown following maternal exercise during pregnancy and lactation (Pinto & Shetty 1995). Exercise during pregnancy (swimming) resulted in low birth weight first generation (F1) pups.

The second generation (F2) offspring of growth retarded F1 animals that were sedentary during pregnancy were also found to be growth retarded, suggesting an adverse intergenerational influence of maternal exercise stress on fetal growth in these animals.

1.4.4.2 Programming of metabolic parameters and blood pressure

Recent studies have been able to demonstrate other intergenerational effects in animal models. Fetal undernutrition can produce effects on glucose homeostasis in a second (F2) generation of rats (Martin et al 2000). Intergenerational effects on birth weight and the endocrine pancreas have also been reported following the use of a low protein, isocaloric diet (Hoet & Hanson 1999).

Intergenerational effects on blood pressure in rabbits has recently been demonstrated in an experiment in which females with surgically induced hypertension were mated with normotensive males (Denton et al 2003). The female offspring of hypertensive rabbits had increased blood pressure as adults when compared with the offspring of sham-operated females, although blood pressure in male offspring was unaffected (Denton et al 2003).

1.4.4.3 Postnatal programming

Postnatal programming may also have intergenerational effects. Overfeeding in the neonatal period has been shown to produce second generation effects on glucose homeostasis (Laychock et al 1995, Vadlamudi et al 1995, Patel et al 2001, Srinivasan et al 2003).

Additionally, post-natal environmental manipulations programming the hypothalamic-pituitary-adrenal (HPA) axis stress response may produce intergenerational effects. A number of studies suggest that differences in behavioural and neuroendocrine responses to stress may be transmitted from one generation to another by non-genomic mechanisms (Meaney 2001). In rodents, naturally occurring variations in maternal behaviour are associated with different HPA stress responsiveness in offspring (Liu et al 1997). Cross-fostering studies have

demonstrated that such differences in maternal behaviour, and therefore differences in offspring stress reactivity, may be transmitted across generations by non-genomic mechanisms (Francis et al 1999). Additionally, postnatal handling of rat pups is associated with programming of the HPA axis; handled offspring show reduced HPA responses to stress and increased maternal care behaviour (Francis et al 1999). Again, these individual differences in maternal behaviour and HPA responses can be transmitted from one generation to another (Francis et al 1999).

1.5 Mechanisms of intergenerational inheritance

From the above, it is clear that intergenerational programming can occur, even in the absence of a continuing environmental stimulus. This would suggest that permanent 'programming' of maternal physiology might lead to the persistence of programming effects across a number of generations. Such effects might be apparently advantageous, as in the secular changes of increasing birth weight across generations which has been documented in some populations (Chike-Obi et al 1996, Skjaerven et al 2000, Kramer et al 2002), or deleterious, as in the perpetuation of low birth weight and higher blood pressure (Barker et al 1989, Walker et al 1998). Why might the environmental experience of one generation affect the offspring of subsequent generations, and how might such intergenerational effects be mediated?

1.5.1 Maternal growth

Exposure of the fetus to an adverse environment *in utero* may lead to permanent alterations in physiology in adulthood. Such physiological changes may result in an adverse intrauterine environment for the offspring of the individual, leading to physiological changes in the next generation and so on. There is evidence that poor maternal intrauterine growth is associated with reduced weight gain during pregnancy (Hackman et al 1983), suggesting that pregnancy may be affected by physiological changes consequent on poor maternal growth. The importance of maternal size in determining the intrauterine growth of offspring has been

demonstrated with cross breeding experiments in Shetland ponies and Shire horses (Walton & Hammond 1938). The offspring were smaller when the Shetland pony rather than the Shire horse was the mother, suggesting that maternal size has an important influence on the size of the offspring. Indeed, short women have small babies (Cawley et al 1954), and British mothers whose stature equalled or exceeded that predicted from midparental height had bigger babies than mothers of smaller stature (Emanuel 1997). Animal studies have shown that organ size is affected by intrauterine malnutrition (Stewart et al 1975), and girls born small for gestational age and remaining small have reduced uterine and ovarian size (Ibanez et al 2000).

However, the growth retarded babies most likely to develop adult cardiovascular risk factors are those who catch up most in terms of growth in childhood. These mothers will not, therefore, be small at the time of conceiving the next generation, suggesting that maternal size is unlikely to account for intergenerational programming of cardiovascular risk.

1.5.2 Socio-economic factors

Socio-economic factors may have a role in intergenerational effects. Lifelong minority status and disadvantage amongst black women in the USA may have played a key part in perpetuating poor intrauterine growth across a number of generations (Collins et al 2002). In two Swedish cohorts born in the 1920s and in 1985, household social class was shown to have a clear influence on birth weight (Vagero & Leon 1994). However, social class is (at least conventionally), as much influenced by paternal as by maternal circumstances, so is unlikely to account for matrilineal inheritance.

Conversely, improvements in the environment and in maternal health could also have intergenerational effects on offspring growth. Secular trends in some populations show increases in mean birth weight of 40 – 100g over decades (Chike-Obi et al 1996, Skjaerven et al 2000, Collins et al 2002). Such a rapid increase in the mean population birth weight provides evidence of the importance of environmental factors in the expression of genetic potential.

1.5.3 Nutrition

One of the major factors proposed to explain fetal programming is maternal undernutrition, and studies in humans and animals suggest that this may have intergenerational consequences. In developing countries, maternal diet can have an effect on birth weight, and may be important in mediating intergenerational effects on birth weight and adult disease. Such effects may also be mediated, or indeed amplified, by adverse environmental conditions persisting across a number of generations. In animal models, second generation effects have been noted following impaired nutrition *in utero*, and exposure during specific time-windows of development may be important (Stewart et al 1975, Stewart et al 1980, Laychock et al 1995, Hoet & Hanson 1999, Martin et al 2000). However, these occur even when nutrition is normal in the F2 generation, so that perpetuation of the insult may not be required to express the effect in later generations.

Early results from the 1944-1945 Dutch famine studies suggested that there may be second generation effects following a specific environmental insult in a previously healthy population (Lumey 1992). Initial studies suggested that mothers with first and second trimester exposure to famine had offspring (F2) of lower birth weights than those not exposed to famine (Lumey 1992). However, this study was flawed in a number of ways, including that birth weights in famine-exposed mothers were not directly ascertained but were extrapolated from another group. A subsequent study found no significant effect of maternal famine exposure on their F2 offspring birth weight (Stein & Lumey 2000).

One small study in Guatemala has demonstrated that nutritional supplementation of girls during early childhood may have a positive effect on the birth weight and height of their offspring (Stein et al 2003), however, as discussed earlier, the role of dietary deficiency in the association between poor fetal growth and later disease in developed countries is unclear. The role of impaired nutrition in intergenerational effects on birth weight and cardiovascular risk therefore remains uncertain, but may be particularly important in maintaining the cycle of intergenerational programming in developing countries.

1.5.4 Glucocorticoids

Animal studies have shown that antenatal glucocorticoids can lead to a number of programming effects, including low birth weight, hypertension, glucose intolerance and elevated plasma glucocorticoid levels (Nyirenda et al 1998, Newnham 2001) and data from human studies suggest that prenatal glucocorticoid exposure lowers birth weight and is associated with increased blood pressure in humans (Doyle et al 2000, Bloom et al 2001). Furthermore, in animal models (Levitt et al 1996, Welberg et al 2000) and in humans (Phillips et al 1998, Levitt et al 2000, Reynolds et al 2001), plasma glucocorticoids are elevated in adults born with lower birth weight. Elevated glucocorticoid levels might potentially mediate both lower birth weight and hypertension through a number of generations.

1.5.5 Blood pressure

Other possible mechanisms underlying intergenerational effects include haemodynamic changes, which may be self-perpetuating in subsequent generations. One such mediator of this effect may be blood pressure; lower maternal birth weight is associated with an increased risk of hypertension during pregnancy (Klebanoff et al 1999) and higher maternal blood pressure during pregnancy is associated with lower offspring birth weight (Churchill et al 1997, Ferrer et al 2000, Brown et al 2001, Buchbinder et al 2002), and with higher offspring blood pressure (Walker et al 1998). In addition, maternal birth weight is related to offspring blood pressure (Barker et al 2000). Although one study found that the association between maternal birth weight and offspring blood pressure was independent of maternal blood pressure later in life (Barker et al 2000), this apparent inherited effect may represent intergenerational influences on fetal programming, with low birth weight and subsequent higher blood pressure influencing fetal growth in such a way as to perpetuate this phenomenon. Indeed this is supported by a recent study demonstrating higher blood pressure in the female offspring of rabbits with secondary hypertension (Denton et al 2003).

1.5.6 Epigenetic mechanisms

Epigenetic modification of the genome is thought to be important in maintaining different patterns of gene expression in different cell groups, in the establishment of parental genomic imprints in germ cells (parental imprinting), and in the erasure of epimutations (Rakyan et al 2001). Epigenetic control of gene expression is likely to be mediated by alterations in DNA methylation and/or modifications of chromatin packaging, possibly via changes in histone acetylation. Both mechanisms may influence transcriptional activity and thus gene expression, mainly by the transcriptional silencing of the modified allele (Rakyan et al 2001). Such modifications influence gene expression, are established early in development and maintained throughout life and may affect the phenotype without changing the DNA sequence, making them prime candidates to explain programming. Epigenetic modification is not restricted to parentally imprinted genes, and the variable expression of some identical alleles within a population may therefore be due to epigenetic modulation rather than genetic differences or adult environmental influences (Rakyan et al 2001).

A number of genes important in modulating fetal growth, particularly those involved in the control of the expression of IGF2, are imprinted; indeed, loss of imprinting of the IGF2 gene in humans leads to Beckwith-Wiedemann syndrome, associated with fetal overgrowth (Morison & Reeve 1998). In addition, IGF2 and a number of related genes are imprinted in the placenta (Young 2001) and it has been proposed that the IGF2 gene and perhaps others may control the placental supply of nutrients to the fetus, and thus affect fetal growth (Constancia et al 2002, Reik et al 2003). In mice, manipulations leading to alterations in the expression of imprinted genes in fetal tissues and in the placenta have been shown to be associated with alterations in fetal growth (Reik et al 2003). Imprinting of genes has been proposed as the mechanism behind the association of lower offspring birth weight with paternal diabetes in the Pima Indian population (Lindsay et al 2000, Lindsay et al 2002a), and in the parental differences seen in the transmission of class III alleles of the variable number tandem repeat minisatellite 5' to the insulin gene (INS-VNTR), variations in which have been associated with type 2 diabetes (Huxtable et al 2000).

The epigenetic silencing of one allele of a gene according to the parent of origin, or parental imprinting, is erased through meiosis and re-established in the offspring. However, recent evidence suggests that epigenetic modifications at some other alleles may not be completely erased during gametogenesis and embryogenesis, potentially resulting in the intergenerational inheritance of the epigenetic state (Roemer et al 1997, Morgan et al 1999). Very recently it has been shown in mice that the variable expressivity of an allele affecting tail development corresponds with differential methylation which shows transgenerational epigenetic inheritance after both paternal and maternal transmission, displays parent of origin effects (the penetrance of the abnormal tail phenotype is greater after paternal transmission) and is influenced by the strain background (Rakyan et al 2003).

Imprinting of genes can be modified by environmental factors (Reik et al 2003) and imprinted genes may be more vulnerable to methylation changes than the rest of the genome (Young 2001). Embryo culture experiments have shown that environmental influences can permanently affect gene expression and have profound effects on growth (Reik et al 1993, Dean et al 1998, Khosla et al 2001, Young et al 2001). Manipulations of the culture medium of the pre-implantation embryo can result in fetal overgrowth in sheep, in association with reduced methylation and a consequent reduction in expression of the IGF2R gene (Young et al 2001). In mice, alterations in the culture medium can result in a reduction in fetal weight associated with reduced expression of IGF2 and H19 (Khosla et al 2001). These effects on fetal growth perhaps represent epigenetic mechanisms. Intriguingly, many growth factors including IGF2, are regulated by glucocorticoids in fetal and adult tissues in vivo and in vitro (Luo et al 1990, Li et al 1993, Miell et al 1994, Mouhieddine et al 1996, Forhead et al 1998, Li et al 2002). Offspring IGFs are also affected by maternal nutrition in rats (Woodall et al 1996a, Petrik et al 1999) and in humans (Barker et al 1993a). Very recently, altered maternal diet during pregnancy has been shown to increase methylation of the agouti gene and alter the phenotype of Agouti Yellow mouse pups. These mice are obese and yellow, due to a mutation in the agouti gene, however pups born to mothers supplemented with vitamins (methyl donors) during pregnancy were found to be thin and brown, and had increased methylation at the agouti locus (Waterland & Jirtle 2003), reducing expression of the mutant allele.

Thus, environmental factors including nutrition and glucocorticoids could potentially influence the expression of genes, affecting fetal growth and later disease risk. If such epigenetic modifications were not erased during gametogenesis and embryogenesis, this could lead to the transgenerational inheritance of 'programmed effects' (Reik et al 2003). Indeed, nutrition mediated epigenetic effects have been proposed as a mechanism to explain the apparent transgenerational inheritance through the male line of cardiovascular disease and diabetes risk described in Swedish men (Kaati et al 2002, Pembrey 2002). Furthermore, evidence is emerging for selective methylation/demethylation of specific promoters of the glucocorticoid receptor (GR) gene in association with variations in maternal care (Weaver et al 2002) which also appear to be inherited.

1.6 The amplification of programming by obesity

The development of obesity appears to be an important factor modifying the risk of later disease associated with low birth weight. Those with low birth weight but who demonstrate catch-up growth in early childhood, appear to be at the highest risk of later obesity (Dietz 1994, Ong et al 2000, Eriksson et al 2003) and those individuals with the lowest birth weight but highest current weight have the highest total body fat (Gale et al 2001). Thus, low birth weight appears to be a risk factor for the development of obesity during childhood and adulthood and a number of human studies have demonstrated that this increases the risk of later disease. In the Preston cohort, insulin resistance was shown to be greatest in those of low birth weight with highest body mass later in life (Phillips et al 1994). In Swedish men, low birth weight was found to be associated with an increased risk of type 2 diabetes and higher blood pressure, with an effect most marked in those who subsequently became obese (Leon et al 1996, Lithell et al 1996). Low birth weight followed by high growth rates after the age of 7 years and a high body mass index (BMI) in childhood is associated with coronary heart disease in men and type 2 diabetes in women in Finland (Eriksson et al 1999, Forsen et al 2000, Eriksson et al 2001). Intriguingly, two patterns of growth were associated with later disease risk, those of low birth weight who remained thin in childhood but became obese as adults had an increased risk of insulin resistance,

whereas those of low birth weight who demonstrated rapid childhood growth, had a high BMI in childhood and who remained obese as adults were at increased risk of developing type 2 diabetes (Eriksson et al 2002, Eriksson et al 2003). In another UK cohort, low birth weight and accelerated weight gain in childhood were associated with the highest adult blood pressure (Law et al 2002).

The long-term effects of malnutrition *in utero* have been studied in the Leningrad Siege and the Dutch Famine Studies. In the Leningrad siege study, a positive association between obesity and blood pressure was found, with a relationship that was strongest in those exposed to malnutrition *in utero* during the siege (Stanner et al 1997, Stanner & Yudkin 2001). Among 19-year-old men exposed to the Dutch famine, those exposed during the first half of pregnancy had a higher risk of obesity than those exposed during later pregnancy (Ravelli et al 1976). Among 50-year-olds, women exposed to famine in early gestation had a higher BMI and waist circumference than unexposed women (Ravelli et al 1999). Furthermore, prenatal exposure to famine is linked to decreased glucose tolerance in adulthood, particularly in those who become obese (Ravelli et al 1998).

The prevalence of obesity, type 2 diabetes and cardiovascular disease is increasing rapidly in urban India (Yajnik 2002). Indian babies are of lower birth weight than Caucasian babies, but have a similar subscapular skin fold thickness, suggesting a lower lean body mass (Yajnik 2002, Yajnik et al 2002). In addition, Indian babies appear to be hyperinsulinaemic (Yajnik et al 2002). In childhood, the highest levels of insulin resistance syndrome variables and total and LDL cholesterol were in those children of low birth weight who had a high fat mass at 8 years (Bavdekar et al 1999). Furthermore, among Indians in rural and urban communities, low birth weight and central obesity in adulthood was found to be associated with increased cardiovascular risk (Yajnik 2002).

The importance of obesity as an amplifier of programming effects has been explored in a number of animal models in which antenatal nutritional manipulations have resulted in offspring of low birth weight and postnatal growth failure. Rats which were exposed to maternal undernutrition throughout gestation, which were then cross-fostered onto normally nourished mothers and subsequently weaned on to a hypercaloric diet demonstrated amplified metabolic and cardiovascular

abnormalities, developing hyperphagia, profound obesity, hyperglycaemia, hyperinsulinaemia, hyperleptinaemia and hypertension (Vickers et al 2000, Vickers et al 2001b). Rats exposed to pre- and postnatal protein restriction which were exposed to a high fat diet at 20 weeks of age developed glucose intolerance as a result of impaired insulin secretion and peripheral insulin resistance, in contrast to those maintained on the low protein diet which demonstrated impaired insulin secretion, but maintained glucose tolerance by increasing peripheral insulin sensitivity. A further control group exposed to a high fat diet only, developed peripheral insulin resistance, but were able to maintain normoglycaemia as a result of increased insulin secretion (Holness & Sugden 1999).

Thus, in humans, adult body size appears to be an important modifying factor in the association between low birth weight and subsequent disease risk, and animal models demonstrate that postnatal dietary manipulations can amplify the metabolic and cardiovascular sequelae of fetal programming.

1.7 Glucocorticoids

Having reviewed the evidence for fetal programming and the potential mechanisms underlying intergenerational effects in humans and animal models, the rest of the introduction will now review glucocorticoid synthesis, secretion and metabolism and consider the evidence that glucocorticoids play a key role in the development of the programmed phenotype. Finally, glucocorticoid action and the role of glucocorticoids in obesity will be discussed.

1.7.1 Synthesis, transport and metabolism

Glucocorticoids are steroid hormones that are synthesised in the mitochondria and endoplasmic reticulum of cells in the zona fasciculata and zona reticularis in the adrenal cortex. All steroid hormones have the same basic structure of three cyclohexane rings and a single cyclopentane ring. Cholesterol, most of which is derived from circulating low-density lipoprotein (LDL) cholesterol, is the precursor for all adrenal steroidogenesis, which involves the action of several enzymes

including a number of the cytochrome p450 family (Figure 1.3). The molecular structures of the major active glucocorticoids (cortisol in man, corticosterone in rats) are depicted in figure 1.3. Over 90% of circulating cortisol and corticosterone are bound to albumin or the high affinity corticosteroid-binding globulin (CBG) (Hammond 1990), an α_2-globulin synthesised in the liver. Only the remaining free circulating hormone is available for transport into tissues and is biologically active. The half-life of these circulating steroids is around 90 minutes, and the metabolism involves a number of steps, which are shown in figure 1.4. Cortisol (corticosterone) and cortisone (11-dehydrocorticosterone) are interconverted by the isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD 1 and 2); their subsequent metabolism follows similar steps. Firstly, the C4-C5 double bond is reduced by either 5α- or 5β-reductase, to give dihydro-metabolites. Cortisol (F) and corticosterone (B) can be metabolised by either 5α- or 5β-reductase, whereas cortisone (E) and 11-dehydrocorticosterone (A), are only metabolised by 5β-reductase. The dihydro-metabolites are then rapidly reduced by 3α-hydroxysteroid dehydrogenase (3α-HSD), to give the tetrahydro-metabolites: in the case of cortisol and cortisone, 5α-tetrahydrocortisol (allo-THF), 5β-tetrahydrocortisol (THF) and 5β-tetrahydrocortisone (THE); and for the rodent steroids, 5α- and 5β-tetrahydrocorticosterone (5α- and 5β-THB) and 5β-tetrahydro-11-dehydrocorticosterone (5β-THA). Further reduction of the 20-oxo group then occurs (by 20α- or 20β-hydroxysteroid dehydrogenase), to yield the cortols and cortolones. Oxidation of these compounds by 21-oxidase yields cortolic and cortolonic acids. In humans, approximately 50% of the secreted glucocorticoids are excreted in the urine as tetrahydro-metabolites, which are conjugated with glucuronic acid to increase water solubility.

Figure 1.3 The adrenocortical steroid biosynthetic pathway.

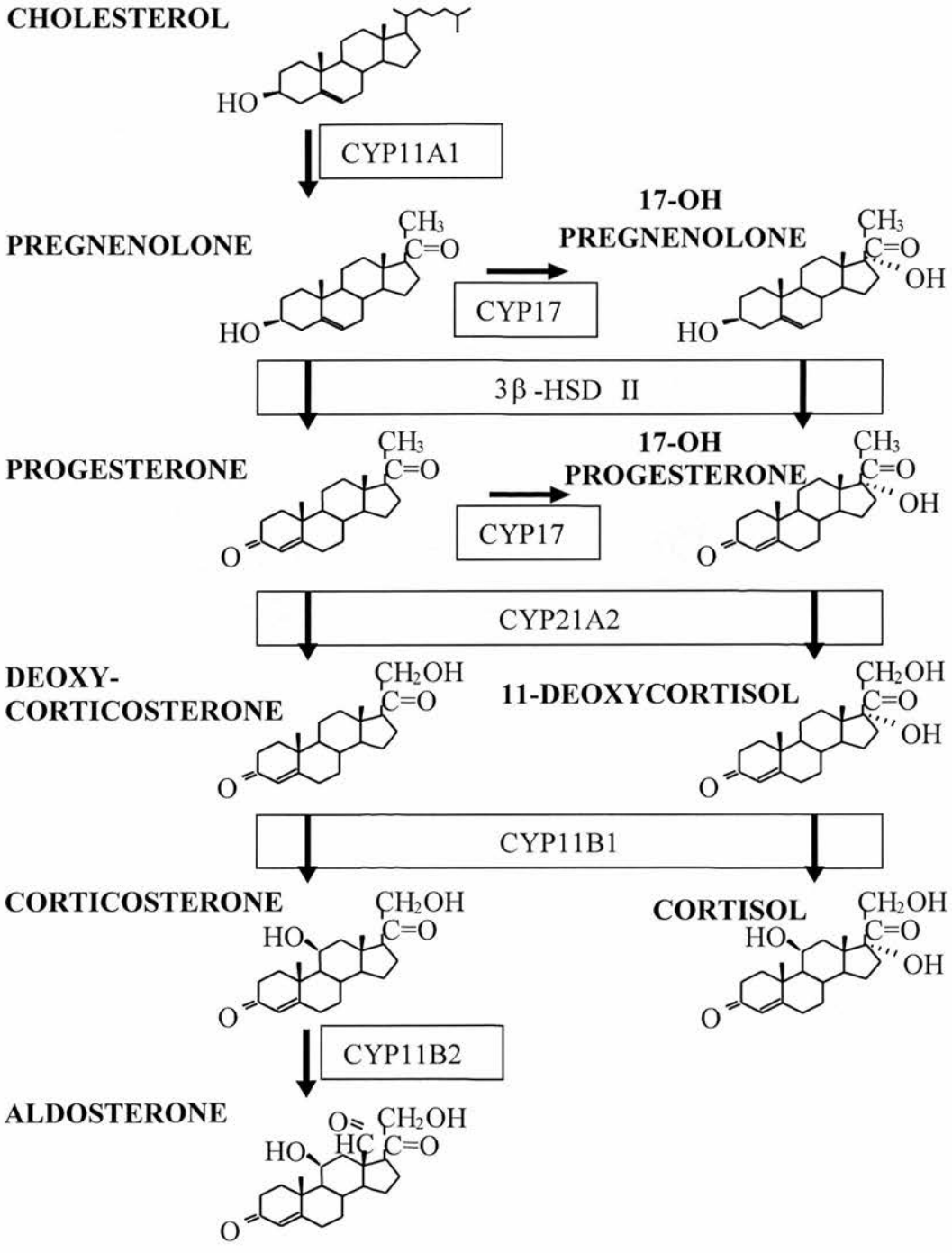
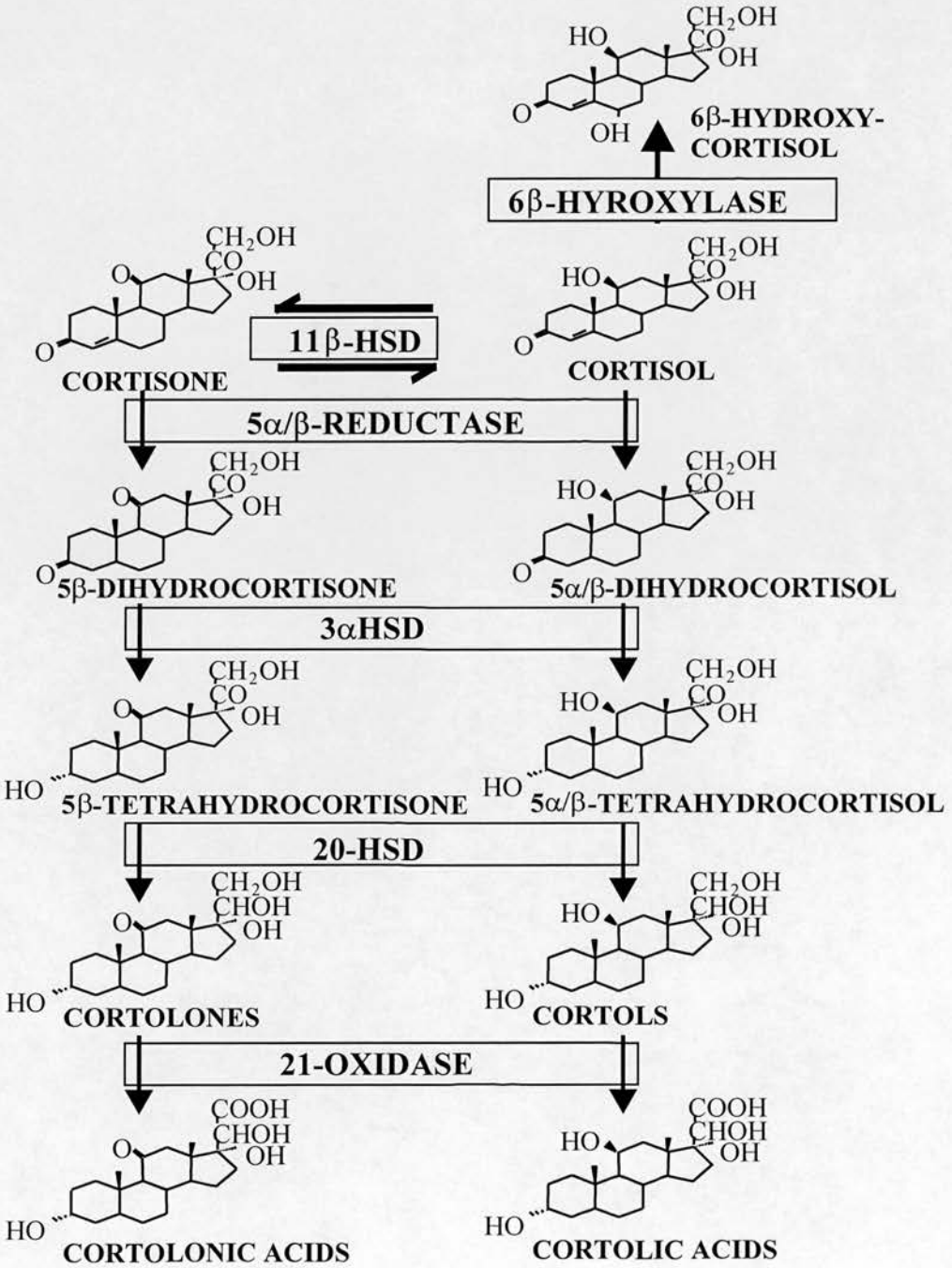


Figure 1.4 Routes of cortisol metabolism *in vivo*

Both 5α- and 5β-reduction of cortisol occur, but cortisone is only acted on by 5β-reductase.

HSD = hydroxysteroid dehydrogenase



1.7.2 The Hypothalamic-pituitary-adrenal (HPA) axis

The secretion of glucocorticoids is regulated by the HPA axis (Figure 1.5). Adrenocorticotrophic hormone (ACTH) is the principal hormone stimulating the production of glucocorticoids in the adrenal. The polypeptide hormone ACTH is produced in the corticotrophs of the anterior pituitary, where it is formed from the cleavage of a larger precursor, pro-opiomelanocortin (POMC). ACTH binds to its receptor on the adrenal cortex cell surface, which is coupled to adenylate cyclase. The subsequent increase in intracellular cyclic adenosine monophosphate (cAMP), leads to steroidogenic acute regulatory (stAR) protein-mediated uptake of cholesterol into mitochondria for steroidogenesis. In addition, chronic stimulation with ACTH leads to an increase in the steroidogenic CYP enzymes (Waterman & Bischof 1997).

The secretion of ACTH is controlled by corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), synthesised in the paraventricular nucleus (PVN) of the hypothalamus. CRH, AVP, ACTH and cortisol are released in a pulsatile manner, with a circadian rhythm, regulated by the suprachiasmatic nucleus of the hypothalamus. In humans, levels are highest before waking and decline through the day; in rats peak levels are achieved in the evening. Glucocorticoids exert a negative feedback effect at the level of the hypothalamus by inhibiting CRH and AVP synthesis in the PVN, and at the pituitary by inhibiting POMC gene transcription (Keller-Wood & Dallman 1984, Davis et al 1986, Eberwine et al 1987, Roberts et al 1987). The regulation of glucocorticoid secretion is also influenced by input from higher centres, particularly the hippocampus (Jacobson & Sapolsky 1991).

Studies in animal models indicate that the HPA axis is an important target for glucocorticoid programming. Prenatal dexamethasone exposure or 11β-HSD 2 inhibition permanently increases basal plasma corticosterone levels in adult rats (Levitt et al 1996, Welberg et al 2001). Both long- and short-term *in utero* exposure to dexamethasone is associated with elevated basal plasma corticosterone levels, however the underlying mechanisms differ depending on the timing and extent of exposure. Exposure to dexamethasone during the last third of pregnancy reduces MR and GR levels in the hippocampus and increases CRH mRNA in the hypothalamic

paraventricular nucleus (PVN) (Levitt et al 1996, Welberg et al 2001). In contrast, dexamethasone throughout gestation does not alter hippocampal GR or MR, but increases receptor expression in the amygdala, a structure which stimulates the HPA axis (Welberg et al 2001). Thus, in the rat, late gestational dexamethasone exposure may permanently alter the 'set point' of the HPA axis at the level of the hippocampus, reducing feedback sensitivity, whereas continuous exposure may increase forward drive of the HPA axis through the amygdala.

In sheep, exposure to betamethasone alters HPA feedback sensitivity (Sloboda et al 2002a), with the outcome depending on the timing of exposure and whether betamethasone was administered to the mother or directly to the fetus (Sloboda et al 2002a). Studies in guinea pigs also demonstrate altered HPA responses after prenatal glucocorticoid exposure (Dean et al 2001, Liu et al 2001), although the effects are smaller, probably because these animals are relatively glucocorticoid resistant because of a mutant GR gene (Keightley & Fuller 1994). Finally, in primates, the offspring of mothers treated with dexamethasone during late pregnancy had elevated basal and stress-stimulated cortisol levels and a 30% reduction in hippocampal size (Uno et al 1994). These studies in rodents, guinea pigs, sheep and primates indicate that exposure to excess glucocorticoids *in utero* can programme HPA axis function. Intriguingly, maternal undernutrition in rats (Langley-Evans et al 1996a) and sheep (Hawkins et al 2000) also affects adult HPA axis function, suggesting that HPA programming may be a common outcome of prenatal environmental challenge, perhaps acting in part via alterations in placental 11β-HSD 2 activity which is selectively down-regulated by maternal dietary constraint (Langley-Evans et al 1996b, Bertram et al 2001).

1.7.3 The 11β-hydroxysteroid dehydrogenase enzymes

The pre-receptor metabolism of glucocorticoids is thought to be an important means of modulating local glucocorticoid action, by regulating the access of hormone to the receptor. The isoenzymes of 11β-HSD catalyse the interconversion of active glucocorticoids (cortisol and corticosterone) and their inactive 11-keto forms (cortisone and 11-dehydrocorticosterone). The 11β-HSDs are microsomal enzymes

that belong to the short-chain alcohol dehydrogenase superfamily (Stewart & Krozowski 1999, Seckl & Walker 2001).

1.7.3.1 11β-hydroxysteroid dehydrogenase type 2

11β-HSD 2 is a high affinity NAD-dependent dehydrogenase that inactivates cortisol and corticosterone. It is principally sited in mineralocorticoid target tissues such as the kidney, colonic mucosa, salivary glands and sweat glands, but is also found in placenta, lung, lymph nodes and vascular endothelium (Stewart & Krozowski 1999). In the kidney, it primarily colocalises with the mineralocorticoid receptor (MR) and acts to protect these receptors from illicit occupation by glucocorticoids, which circulate at much higher concentrations than mineralocorticoids and bind with equal affinity. 11β-HSD 2 is also present in the placenta (Stewart et al 1995, Brown et al 1996a, Waddell et al 1998), where it is thought to be important in protecting the fetus from the high circulating concentrations of maternal glucocorticoids.

1.7.3.2 11β-hydroxysteroid dehydrogenase type 1

11β-HSD 1 is a lower affinity NADP(H)- dependent enzyme, which is found in many tissues, including brain, liver, adipose tissue, lung, vascular smooth muscle, gonads and the adrenal cortex (Stewart & Krozowski 1999). Original investigations found this enzyme to be bi-directional, with both reductase and dehydrogenase activity, although the reductase activity was unstable *in vitro* (Lakshmi & Monder 1988). More recent studies have demonstrated that the enzyme is predominantly a reductase when studied in intact cells or tissues *in vitro* or *in vivo,* but the dehydrogenase direction is preferred when cells are disrupted (Jamieson et al 1995, Rajan et al 1996, Bujalska et al 1997, Ricketts et al 1998, Jamieson et al 2000). Isolated rat liver models demonstrate that the 11β-HSD 1 enzyme is a predominant 11β-reductase, and regenerates active glucocorticoids from their inactive metabolites (Jamieson et al 2000). It has therefore been hypothesised that 11β-HSD 1 is important in maintaining local concentrations of glucocorticoid, in particular in those tissue in which glucocorticoids play important roles in metabolic regulation. There is

some evidence to support this hypothesis; inhibition of 11β-HSD 1 by chronic oestrodiol administration inhibits the expression of glucocorticoid-induced gluconeogenic enzymes (Jamieson et al 1999b) and transgenic mice with a targeted disruption of the 11β-HSD 1 gene are unable to convert 11-dehydrocorticosterone to corticosterone following adrenalectomy (Kotelevtsev et al 1997). Furthermore, these 11β-HSD 1 knockout mice have a phenotype compatible with impaired glucocorticoid regeneration despite elevated basal corticosterone concentrations; they have impaired induction of PEPCK and glucose-6-phosphatase on fasting and resist hyperglycaemia on exposure to stress or obesity (Kotelevtsev et al 1997).

Factors influencing 11β-HSD 1 include glucocorticoids (Voice et al 1996), CRH and ACTH (Friedberg et al 2003), cytokines including TNFα (Tomlinson et al 2001, Friedberg et al 2003), thyroid hormones (Whorwood et al 1993), sex steroids and GH (Low et al 1993, Low et al 1994), IGF-1 and insulin (Voice et al 1996, Whorwood et al 2001). Chronic stress or elevated glucocorticoid levels are associated with reduced 11β-HSD 1 activity (Jamieson et al 1999a). In addition, both PPARα and PPARγ agonists attenuate 11β-HSD 1 activity (Hermanowski-Vosatka et al 2000, Berger et al 2001).

In fetal sheep, exposure to excess glucocorticoid increases hepatic 11β-HSD 1 mRNA and protein (Yang et al 1995, Sloboda et al 2002b), however studies in the rat have not shown any alterations in 11β-HSD 1 activity in the liver of adult rats exposed to dexamethasone *in utero* (Nyirenda et al 1998) and antenatal carbenoxolone treatment reduces 11β-HSD 1 in adult animals (Saegusa et al 1999).

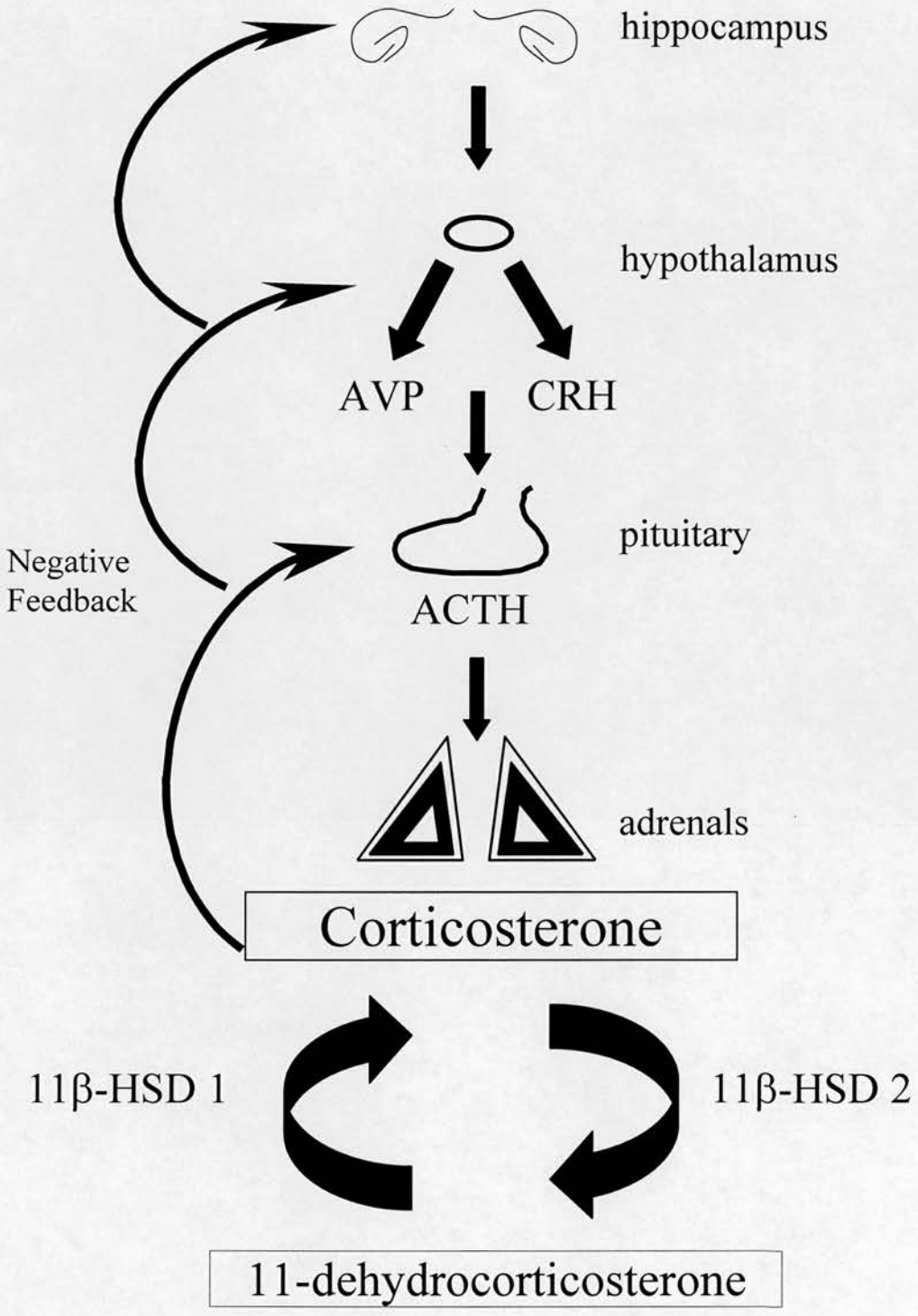


Figure 1.5 The HPA axis in rodents, depicting sites of negative feedback. CRH = corticotrophin releasing hormone; ACTH = adrenocorticotrophic hormone; AVP = arginine vasopressin

1.7.4 The glucocorticoid receptor

The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. These have a common functional domain structure comprising a variable amino-terminal (N-terminal) region, a central DNA binding domain; important for interaction with specific target gene sequences, DNA induced homodimerisation and transcriptional activation, and a carboxy-terminal (C-terminal) ligand-binding domain. The N-terminal and C-terminal domains have additional transcription activation functions; the ligand-binding domain also has a hormone-dependent dimerisation function and a hormone dependent nuclear localisation signal.

The human GR (hGR) gene contains 9 exons; exon 1 and the first part of exon 2 contain the 5' untranslated region (5'UTR) and exons 2-9 contain the coding sequences (Encio & Detera-Wadleigh 1991). Three different hGR mRNAs have been identified. GRα and GRβ both contain exons 1-8 but have different versions of exon 9 as a result of alternative splicing and encode the hGRα and hGRβ receptor isoforms (Hollenberg et al 1985); a third hGR mRNA is thought to encode hGRα. Human GRα and GRβ protein differ after amino acid 727 and in addition, alternative translation initiation results in two different isoforms (A and B), of hGRα (Yudt & Cidlowski 2001).

In the absence of ligand, GRα exists in a cytoplasmic complex with other proteins including hsp90. On ligand binding, GRα dissociates from this complex and translocates to the nucleus, where the receptor-ligand complex undergoes dimerisation and binds to specific DNA sequences known as glucocorticoid-responsive elements (GREs) in the promoter region of target genes. Binding of GRα to GREs leads to transcription initiation, however the mechanisms of this are not fully understood. The transcriptional activity of GRα also depends on interaction with other transcription factors (Gottlicher et al 1998) and various co-activators and co-repressors (Sheppard et al 1998, Collingwood et al 1999, Glass & Rosenfeld 2000, Jenkins et al 2001). Such interactions facilitate recruitment of the basal transcription machinery and/or lead to chromatin remodelling, leading to activation or repression of target genes. Many glucocorticoid responsive genes have now been

identified, including many involved in carbohydrate and lipid metabolism (Stewart 2003).

Recently there has been increasing interest in the possibility that very rapid corticosteroid effects may be mediated by membrane bound or associated forms of GR, however the mechanisms of such effects remain to be determined (Iwasaki et al 1997, Chen et al 1999).

1.7.5 Regulation of GR expression

Glucocorticoid receptors are expressed in most cells, however expression and sensitivity to glucocorticoids varies between individuals, tissues and cell types (Herman et al 1989, Lim-Tio et al 1997). The level of expression of GR is critical for cell function, indeed, transgenic mice with a reduction of 30-50% in tissue levels of GR have major neuroendocrine, metabolic and immunological abnormalities (Pepin et al 1992, King et al 1995). In the rat, prenatal dexamethasone exposure leads to tissue-specific alterations in GR, which are thought in part, to explain the adult phenotype of the dexamethasone-programmed animal; GR expression is permanently increased in the periportal zone of the liver, in the amygdala, in adipose tissue and in some muscle fibre types (Nyirenda et al 1998, Welberg et al 2001, Cleasby et al 2003a), but reduced in the hippocampus (Levitt et al 1996, Welberg et al 2001). In addition to its interactions with transcription factors, co-activators, co-repressors and a number of cytosolic proteins, a number of potential mechanisms exist for the regulation of GR expression. Binding sites for a number of transcription factors have been identified in the GR promoter region, which may permit cell type- and tissue specific regulation of GR (reviewed in Yudt & Cidlowski 2002) and GR may undergo post-translational modification (Yudt & Cidlowski 2002). Glucocorticoids themselves appear to regulate GR expression, possibly through effects on transcription, mRNA stability and protein stability (Schaaf & Cidlowski 2003). In addition, GRβ is thought to act as a dominant-negative inhibitor of GRα (Bamberger et al 1995, Oakley et al 1996, Oakley et al 1999), although its role in disease or glucocorticoid resistance remains unclear (Schaaf & Cidlowski 2003).

Tissue-specific expression patterns may also be achieved by the use of alternate promoters (McCormick et al 2000, Breslin et al 2001). Multiple translation initiation sites have been identified in exon 1 in rats (McCormick et al 2000), mice (Strahle et al 1992) and humans (Breslin et al 2001). These give rise to a number of GR mRNAs with different 5' untranslated regions within exon 1, which are spliced onto the common translated sequence beginning at exon 2. In the rat, two of the alternate exons are present in all tissues which have been studied, however others are tissue-specific (McCormick et al 2000). This permits considerable complexity of tissue-specific variation in the control of GR expression, indeed, first exon usage appears to be altered by perinatal environmental manipulations (McCormick et al 2000).

1.7.6 Effects of glucocorticoids

1.7.6.1 Effects of glucocorticoids on metabolism

Glucocorticoids act to increase blood glucose concentrations by a number of mechanisms. Cortisol stimulates glycogen synthesis by stimulating glycogen synthase and inhibiting glycogen breakdown and additionally, increases hepatic gluconeogenesis by stimulating the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. In addition, peripheral uptake and utilisation of glucose by muscle and fat is reduced. Glucocorticoids also have a permissive effect on the action of other hormones including catecholamines and glucagon.

Glucocorticoids stimulate lipolysis, with the release of free fatty acids and glycerol. Adipocyte differentiation is stimulated through the transcriptional activation of a number of key genes, and in situations of glucocorticoid excess, this results in a predominant increase in visceral fat, possibly due to the increased expression of GR and 11β-HSD 1 in this fat depot.

1.7.6.2 *Effects on blood pressure*

Glucocorticoids act to increase blood pressure by a number of mechanisms. They have a permissive effect to potentiate the vascular response to catecholamines, (particularly noradrenaline) and angiotensin II. Glucocorticoids inhibit nitric oxide synthase, thereby reducing nitric oxide-mediated endothelial dilatation and act in the distal nephron of the kidney to promote sodium and water retention.

1.7.6.3 *Growth and development*

Glucocorticoids accelerate the development of a number of organs in the developing fetus and are widely exploited in obstetric practice to accelerate lung development in threatened preterm labour. However, glucocorticoids in excess inhibit linear growth probably as a result of their catabolic effects on bone, muscle and connective tissue and inhibition of the effects of IGF-1. In bone, glucocorticoids inhibit osteoblast function and IGF-1 generation, while osteoclast numbers increase; they also inhibit the generation of matrix collagen and its mineralisation. Glucocorticoids inhibit calcium absorption in the gut, while increasing renal calcium excretion by interfering with vitamin D action. In muscle, glucocorticoids are associated with atrophy of type II muscle fibres, and in connective tissue with reduced collagen synthesis. Glucocorticoid treatment during pregnancy reduces birth weight in animals (Reinisch et al 1978, Ikegami et al 1997, Nyirenda et al 1998, Newnham et al 1999, Newnham 2001) and in humans (French et al 1999, Bloom et al 2001).

1.7.6.4 *Effects on the brain*

Glucocorticoids are essential for normal brain development and both GR and MR are expressed in many parts of the brain including the hippocampus, hypothalamus, cerebellum and cortex (McEwen et al 1986). Glucocorticoid excess can have a number of adverse effects on cells in the CNS, particularly in the hippocampus. These effects include disruption of synaptic plasticity, atrophy of dendritic processes and, under some circumstances, cell death (Sapolsky 1999). In addition,

glucocorticoid excess has a number of psychological effects including depression, lethargy and psychosis.

1.7.6.5 Other effects

Glucocorticoids have many other important effects including anti-inflammatory actions and immunomodulatory functions. Glucocorticoids also have effects on the gut, and a number of other endocrine systems.

1.7.7 Glucocorticoids and obesity

Excess circulating glucocorticoids are associated with obesity, particularly abdominal obesity. In Cushing's syndrome, exposure to elevated circulating glucocorticoids results in the accumulation of visceral fat, cardiovascular complications and a number of metabolic sequelae, including glucose intolerance, insulin resistance, diabetes and hyperlipidaemia. Obesity in humans is associated with a similar pattern of cardiovascular risk factors; a cluster of features termed the 'metabolic syndrome'. Additionally, increased cortisol concentrations in blood, saliva and urine are associated with hypertension, glucose intolerance, insulin resistance and hyperlipidaemia (Phillips et al 1998, Phillips et al 2000).

Such findings have prompted a number of studies that have sought to determine whether abnormalities in glucocorticoid secretion, action or metabolism may underlie the development of visceral adiposity and the metabolic syndrome associated with obesity. Much work to characterise alterations in glucocorticoid metabolism associated with obesity has been carried out in animal models. Obese Zucker rats, which are leptin resistant due to a homozygous point mutation in the leptin receptor gene, have been used to explore the changes in glucocorticoid metabolism associated with obesity. Obese Zucker rats are hypercorticosteronaemic, hyperinsulinaemic, insulin resistant and have HPA axis activation (Livingstone et al 2000a, Mattsson et al 2003). Recent studies have shown that there are tissue-specific alterations in glucocorticoid metabolism in these animals; in the liver, 11β-HSD 1 is decreased, whereas 5α-reductase is increased, suggesting increased hepatic

glucocorticoid inactivation. In contrast, in visceral adipose tissue, the activity of 11β-HSD 1 is increased, which may enhance local glucocorticoid reactivation and promote obesity (Livingstone et al 2000a). Furthermore, decreased reactivation of corticosterone by 11β-HSD 1 in the hippocampus may exacerbate the HPA axis activation (Mattsson et al 2003). In addition, a number of studies have demonstrated altered peripheral and central GR and MR expression in the obese Zucker rat. Recently it has been shown that MR expression is decreased in the hippocampus in obese rats (Mattsson et al 2003). Mineralocorticoid receptors mediate glucocorticoid feedback in the basal state, and this decrease in expression may further explain the HPA activation observed in the obese animal. Other studies have demonstrated changes in peripheral levels of GR, reporting normal or reduced hepatic GR number and affinity (Shargill et al 1987, White & Martin 1990, Langley & York 1992).

In such animal studies, obesity and features of the metabolic syndrome appear to be glucocorticoid dependent, adrenalectomy or treatment with glucocorticoid receptor antagonists reverses the obese phenotype in obese ob/ob mice and obese Zucker rats (Yukimura et al 1978, Shimomura et al 1987, Langley & York 1990).

In humans most studies suggest that peak plasma cortisol levels are not elevated in idiopathic obesity (Phillips et al 1998, Rosmond et al 1998, Rask et al 2001, Reynolds et al 2001), although there is evidence for increased cortisol secretion (Marin et al 1992, Pasquali et al 2002). Altered peripheral glucocorticoid metabolism has been demonstrated in human subjects, with enhanced inactivation by 5α-reductase (Andrew et al 1998), impaired reactivation by hepatic 11β-HSD 1 (Rask et al 2001, Rask et al 2002) and enhanced reactivation in adipose tissue (Rask et al 2001, Rask et al 2002, Lindsay et al 2003).

1.8 Aims of this thesis

The aim of this thesis was to study the intergenerational effects of programming by *in utero* exposure to excess glucocorticoid in the rat and to delineate potential underlying mechanisms. We also sought to investigate whether the programming phenotype was amplified by obesity. The following list of aims was addressed in this thesis:

1. To study whether dexamethasone programming in rats is associated with any effects on birth weight, PEPCK and glucose tolerance in subsequent generations without any further manipulation.

2. To investigate the influence of maternal and paternal phenotypes on intergenerational programming.

3. To develop a model of high fat feeding in which to study the effects of obesity on the programming phenotype.

4. To study the effects of obesity on the phenotype of the dexamethasone-programmed rat.

Chapter Two – Materials and Methods

2.1 Materials

2.1.1 General Chemicals

Ethanol	Hayman Ltd., Witham, Essex, UK.
Formamide	
Formaldehyde	
Other organic solvents	BDH-Merck, Poole, Dorset, UK.
Malate Dehydrogenase	Roche Diagnostics Ltd., Lewes, East Sussex, UK.
All other solid chemicals	Sigma-Aldritch Ltd., Poole, Dorset, UK.

Enzymes were purchased from Promega, Southampton, UK.
HPLC grade solvents were obtained from Rathburn Chemicals, Walkerburn, UK.
Radioactivity was obtained from Amersham, Bucks, UK.
Sources other than these are indicated in parenthesis.

2.1.2 Molecular Biologicals

TaqMan™ primers and probes	
TaqMan™ PCR core reagent kit	Applied Biosystems, Applera, Warrington, Cheshire, UK.
NICK sephadex G-50 DNA column	Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK.
Zeta-Probe® GT, Nylon membrane	Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK.

Agarose	Biowhittaker Molecular Applications, Wokingham, Surrey, UK.
PicoFluor 40 Scintillant fluid	Canberra Packard, UK.
TRIzol®	Invitrogen Life Technologies/Gibco BRL, Paisley, UK.
Random Primed DNA Labeling Kit	Roche Products Ltd., Welwyn Garden City, Hertfordshire, UK.
Kodak Biomax MS film Kodak Biomax MR film	Sigma.

2.2 Equipment

Ultra-Turrax T8 auto-homogeniser	Ika Labortechnik, Staufen, Germany
Dri-block DB Series	Techne, Cambridge, Cambridgeshire, UK
GeneQuant RNA/DNA Calculator	Amersham Pharmacia, Biotech
1450 Microbeta Plus Liquid Scintillation Counter (used for standard cpm counting)	Wallac Oy
Eppendorf Centrifuge 5415C (used for <2ml volume Eppendorf tubes)	Eppendorf AG, Hamburg, Germany
Labofuge 400R Centrifuge (used for 15 and 50 ml volume Falcon and Corning tubes)	Heraeus, Brentwood, Essex, UK
Optima™ TLX Ultracentrifuge (rpm>14000)	Beckman Instruments, High Wycombe, Buckinghamshire, UK

UV-160A UV-Visible recording Spectrophotometer	Shimadzu Europa, Milton Keynes, Buckinghamshire, UK
EL 312e Bio-kinetics Microplate reader	Bio-Tek Instruments Inc, Winooski, Vermont, USA
Phosphorimager FLA-2000	Raytest Scientific Ltd
Phosphorimager screens	Fuji Photo Film Company Ltd, Tokyo Japan
TLC plates	TLC Aluminium sheets Merck, Darmstadt, Germany
Whirlimixer	FSA Laboratory Supplies, Fisons plc, UK
Water Bath	Grant Instruments, Cambridge Cambridgeshire, UK
Hyperprocessor	Amersham Life Sciences
Gel Documentation system	Appligene, Oncor, UK
PH meter	Denver Instruments
Microvette tubes (CB 300)	Sarstedt, Leicester, UK
Electrophoresis Power Pac 300	Bio-Rad Laboratories Ltd.
Eppendorf Mastercycler gradient (PCRs, RTs and RNA denaturation)	Eppendorf AG, Hamburg, Germany.

2.3 Software

Muticalc Advanced v2.1	Wallac Oy
Fujifilm Fluorescent Image Analyser FLA-2000 V.1.0 Aida 2.0 Auto Image Data Analyser	Raytest Scientific Ltd, Sheffield UK
Statistica v 5.0	Statsoft, Tulsa, Oklahoma, USA

2.4 Standard solutions

Ultra-pure water	dH_2O, UV-treated and autoclaved
DEPC-treated water	0.5ml DEPC was added to 500ml deionised water, agitated and left to stand for 1-24 hours before autoclaving.
Borate buffer	8.25g boric acid and 2.7g Sodium hydroxide were dissolved in 1l dH_2O and set to pH 7.4 with hydrochloric acid.
Deionoised formamide	150ml formamide was mixed with 15g mixed bed ion-exchange resin for >1 hour, filtered twice and stored away from light.
10x MOPS buffer	0.2M 3-[N-morpholino]propanesulfonic acid, 50mM Na acetate, 5mM EDTA, pH7. Autoclaved before use.

10 x TBE buffer	104.9g TRIS, 55.7g boric acid and 4.7g sodium EDTA were made up to 1l with deionised water and autoclaved.
Phosphate buffer	13.8g sodium dihydrogen phosphate (0.2M), 42.6g disodium hydrogen phosphate (0.6M), 0.93g EDTA were made up to 500ml with deionised water and autoclaved.
20 x SSC	350.64g sodium chloride and 176.46g sodium citrate were made up to 2l with deionised water, set to pH 7.4 with hydrochloric acid and autoclaved.
TE buffer	10mM tris, 1mM EDTA, adjusted to pH 8 with hydrochloric acid. Autoclaved before use.
Loading buffer for Northern Blot	50% glycerol, 1mM EDTA, 0.25% Bromophenol blue, 0.25% xylene cyanol.
Homogenisation buffer	100g glycerol solution, 300mg Tris, 186mg EDTA made up to 500ml with dH_20, set to pH 7.5 and stored at 4°C. Just prior to use 7.7mg DTT was added to a 50 ml aliquot.
C Buffer	63g glycerol, 8.77g sodium chloride, 186mg EDTA, 3.03g Tris made up to 500ml with dH_20 and set to pH 7.7.

Hepes Sucrose buffer for PEPCK assay	42.8g sucrose and 0.6g Hepes made up to 500ml with dH_20 and set to pH 7.4. Stored at 4°C.
Hepes Sucrose buffer for 5β-reductase assay	42.8g sucrose and 1.2g Hepes made up to 500ml with dH_20 and set to pH 7.5. Stored at 4°C.
5β-reductase assay buffer	10.95g sucrose (320mM) and 15.4mg DTT (1mM) made up to 100 ml with 40mM sodium phosphate buffer set to pH 7.4. Made up fresh prior to use.
Kreb's Ringer Bicarbonate Buffer	118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$, pH 7.4. Stored at 4°C and supplemented with 0.2% glucose immediately prior to use.

2.5 Animal maintenance

Wistar rats were supplied by Charles River UK Ltd, Margate, Kent, UK. Females were purchased for mating at 200-250g, along with males of equivalent age.

Feed (Standard rat chow) was supplied by Special Diets Services, Witham, Essex, UK, and contained 61.9% carbohydrate, 18.8% protein, 3.4% oil and 0.6% salt.

High fat and Control diets were supplied by Research Diets Ltd, New Brunswick, New Jersey, USA. Contents are detailed in Section 2.20.

All animal procedures were carried out under the terms of the Animals (Scientific Procedures) Act 1986 and Project Licence number 60/2466. William Mungall, Keith Chalmers, Donald Hay and Sharon Rossiter were responsible for the maintenance of, and prenatal treatment of rats. Animals were under the primary care of the animal research technicians at the Biomedical Research Facility throughout the experimental

period. All rats were maintained under conditions of controlled lighting (lights on 0700 to 1900 hours daily) and temperature (22°C), with *ad libitum* access to food and water. Adult and post weaning rats were kept five to a cage except where otherwise stated and cleaned out weekly.

2.5.1 Production and care of offspring

Rats were allowed to acclimatise for two weeks after arrival in the animal unit, before mating, at which time a single virgin female was housed with a male in a breeding cage. The day on which the expelled vaginal plug was noted was designated day zero of pregnancy (E0); females were then housed singly throughout pregnancy until delivery, which occurred on days 20 to 22. Litters were weighed individually at birth and culled to eight, leaving males and females. Pups were weaned at three weeks of age and housed with littermates or with same aged pups from the same treatment group. In all cases experimental cohorts included males and females selected randomly from as many litters as possible.

2.5.2 Prenatal administration of dexamethasone

Pregnant females were injected subcutaneously with a solution of 100μg per kg dexamethasone in 0.9% saline containing 4% ethanol (F1 dex mothers) or with an equivalent volume of vehicle (1ml/kg) (F1 veh mothers) at the same time each morning between days 15 and 21 of pregnancy inclusive.

2.5.3 Culling and harvesting of tissues

Animals were killed by cervical dislocation, CO_2 or decapitation, between 9am and 12pm, without prior withdrawal of food unless stated. Tissues were dissected, weighed where appropriate and snap frozen on dry ice. Tissues were stored at -80°C. Trunk blood was collected into plastic tubes containing 1ml 100mM EDTA through an EDTA coated funnel and placed on wet ice. These samples were centrifuged as soon as possible at 200g for 10 minutes at 4°C and the supernatant plasma aliquotted into Eppendorf tubes and stored at -20°C.

2.6 Glucose Tolerance Tests

Rats were fasted for 17 hours prior to oral glucose tolerance testing. Commencing at 9am, rats were weighed. The tails were nicked with a scalpel towards the distal end and the basal blood sample was collected into a Microvette CB 300 tube with EDTA (Sarstedt). 2g/kg glucose solution (0.5g/ml) was administered by gavage, and the rats were replaced in their cages. Further samples were taken from the same tail nick by massaging the tails at 30 and 120 minutes after administration of glucose. Approximately 300µl blood was taken at each time point, and centrifuged as before for collection of plasma, before storage at -20°C.

2.7 Measurement of plasma insulin and leptin concentrations

The concentration of insulin in plasma samples was measured using Crystal Chem® rat insulin ELISA and ultrasensitive rat insulin ELISA kits (Crystal Chem Inc., Chicago, Illinois, USA) according to the manufacturer's instructions. The standard kits measure insulin concentration in the range of 156 to 10000pg/ml; intra- and inter-assay CVs are reported as 3.5 and 6.3%. The ultrasensitive rat insulin ELISA kit measures insulin concentration in the range of 100 to 6400 pg/ml; intra- and inter-assay CVs are reported as 5.5% and 4.8%. Leptin concentrations were measured in trunk plasma using Crystal Chem® mouse leptin ELISA kit. The manufacturer reports that the anti-mouse antibody cross reacts 90% with rat leptin. The mouse leptin ELISA kit measures leptin concentration in the range of 200 to 12,800 pg/ml; intra- and inter-assay CVs are reported as 5.4% and 6.9%.

2.8 Measurement of plasma glucose concentrations

This assay was performed on samples taken during oral glucose tolerance testing, using the Sigma Infinity™ glucose reagent. Hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate. The product is then oxidised to 6-phosphogluconate, with the generation of NADH. The amount of NADH formed is proportional to the amount of glucose present, and was measured by the increase of UV absorbance of the reaction mixture at 340nm. The UV absorbances of 10µl plasma sample or glucose standard (0-44mmol/l) were then measured in duplicate in

a single assay after the addition of 1500 µl reagent and 5-60 minutes incubation at room temperature. Standard curves of [glucose] versus UV absorbance units were used to calculate the glucose concentrations of the sample. The inter- and intra-assay coefficients of variation were <2%.

2.9 Measurement of plasma lipid parameters

These measurements were made by Dr Philip Wenham, Department of Medical Biochemistry, Western General Hospital, Edinburgh.

Automated assays based on a colourimetric end-point were used, utilising kits obtained from Wako Pure Chemical Industries Ltd, Osaka, Japan (Non-Esterified Fatty Acids) or Roche Diagnostics Ltd (Total and High Density Lipoprotein Cholesterol and Triglycerides).

2.10 Measurement of plasma corticosterone concentration

Plasma corticosterone concentration was measured by radioimmunoassay. The assay was developed by Dr Chris Kenyon, who reported intra- and inter-assay variations of 9.4 and 9.2% respectively, and cross-reactivities for progesterone, deoxycorticosterone and cortisol of 7.7, 6.5 and 5.3% respectively, compared to corticosterone (100%).

Plasma was diluted 1:10 in borate buffer containing 0.5% BSA, and heated to 75-80°C for 30 minutes to destroy corticosterone-binding globulin.

^{3}H-corticosterone was suspended in borate buffer to give between 10000 and 15000cpm per 50µl solution on the β-counter. Rabbit anti-corticosterone antibody was added to this mix in a 1:100 dilution.

20µl aliquots of diluted plasma sample or corticosterone standard (320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0nM corticosterone in borate buffer) were incubated in duplicate with 50µl ^{3}H-corticosterone/primary antibody mix in 96 well plates.

50µl scintillation proximity assay beads (SPA; Amersham, Bucks, UK) diluted with borate buffer was then added to each well, the plates were then sealed, shaken and left at room temperature overnight (<24 hours) to equilibrate. Plates were counted on the β-counter and the concentration of corticosterone in each sample was calculated

from a graph of cpm versus [corticosterone] generated using the Multicalc programme.

2.11 Measurement of blood pressure by carotid cannulation

These measurements were performed by Dr Patrick Hadoke of the Department of Endocrinology. Briefly, cannulae were placed in a carotid artery under anaesthesia, flushed with 0.2ml heparin (20 units/ml), and the animal allowed to recover for 12-24 hours. On the day following surgery (day 1), the cannula was flushed with 0.2 units heparin. Blood pressure was measured on days 2 and 3 (cannulae were almost always found to be blocked by day 4), after connecting the cannula to a pressure transducer. Blood pressure was measured with the animal in a quiescent state and unrestrained, over a period of 5 minutes.

2.12 Extraction of total RNA from tissue

2.12.1 Liver

RNA was extracted with TRIzol®, using a method derived from that of Chomczynski & Sacchi (Chomczynski & Sacchi 1987). Approximately 100mg of liver was homogenised in 1 ml TRIzol®. The mixture was allowed to stand for 5 minutes at room temperature to permit the dissociation of nucleoprotein complexes. 200µl of chloroform per 1 ml of TRIzol® was added and the tubes shaken for 15 seconds. The tubes were centrifuged at 14000g at 4°C for 15 minutes and the upper aqueous phase transferred to another Eppendorf. 0.5ml propan-2-ol per 1ml TRIzol® was added and the mixture vortexed. The tubes were incubated at room temperature for 10 minutes to precipitate the RNA and then spun at 14000g for a further 10 minutes to pellet the RNA. The supernatant was discarded and the pellet washed by vortexing with ≥ 1 volume 75% ethanol. The tubes were then centrifuged at 7500g for 5 minutes at 4°C. The ethanol was removed by drawn-out pasteur pipette and the pellet air-dried before resuspending in DEPC-treated water. Resuspended RNA from the same tissue sample was recombined in a single tube.

2.12.2. Adipose tissue (except omental adipose tissue)

As for liver, except that the tissue was first homogenised in TRIzol®, involving an average of three 10 second bursts at the three-quarter speed setting, and centrifuged at 2000g for 3 minutes at 4°C. The upper lipid layer was discarded, and the subnatant aliquotted into Eppendorf tubes for RNA extraction.

2.12.3 Omental adipose tissue

Only small amounts of omental adipose tissue were available for RNA extraction, therefore the RNAID matrix (Anachem, Luton, UK) was used. This effectively binds RNA, sequestering it away from possible RNAse degradation. Using this system, RNA can be eluted in high concentrations and purity with washes that remove impurities without loss of bound RNA. Following homogenisation in TRIzol®, and separation from the upper lipid layer as described above, RNA was extracted as follows. 250µl of chloroform was added, samples were vortexed, incubated at room temperature for 2 minutes then on ice for 15 minutes. Samples were then centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous phase was transferred to another Eppendorf. To this was added 20µl of resuspended RNAID Matrix, followed by vortexing. Samples were spun at 14000g for 1 minute. The supernatant was removed and discarded. The pellet was washed with 500µl wash buffer (RNA wash; Anachem) followed by centrifugation at 14000g to produce a pellet. After repeating the wash step twice more, the wash buffer was removed and the pellet resuspended in 18µl DEPC- treated water with RNase inhibitor and DTT (89µl DEPC- water, 10µl 100mM DTT, 1µl RNase inhibitor).

2.13 Quantitation and agarose gel electrophoresis of extracted RNA

1µl of resuspended RNA from each sample was diluted with 99µl DEPC-treated water and its UV absorbance at 260 and 280nm measured on the GeneQuant. The RNA concentration in the sample was calculated from this. The ratio of Absorbance (260nm) / Absorbance (280nm) was also recorded as an indication of the purity of the RNA. Values close to 2.0 are desirable.

Agarose gel electrophoresis was used to ensure that the RNA was intact. A 30ml 1% agarose gel was used containing 3ml 10x TBE and 0.5µl ethidium bromide using RNase-free materials. 0.5-1.0µg of RNA was loaded in 2.5µl loading buffer per sample, and electrophoresis performed in 0.5 x TBE. The gel was run at 60mA for approximately 40 minutes and the results viewed on the trans-illuminator at 240nm. The presence of intact 28S, 18S and 5S Ribosomal RNA bands indicated that the preparation was undegraded.

2.14 Protein concentration

Protein concentration of tissue homogenates and microsomal preparations was determined colorimetrically using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hampstead, UK). Samples were diluted with buffer to give solutions in the linear range of 0.05-1.4 mg protein/ml, and BSA standards were prepared in the same buffer to 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mg/ml. 25µl BIO-RAD protein assay solution A and 200µl BIO-RAD protein assay solution B were added to duplicate 10µl aliquots of samples and standards in a 96 well plate. After 15 minutes incubation at room temperature, the absorbance in each well was measured at 750nm in a microplate reader. The concentration of protein in each sample was calculated from the mean absorbance, the dilution factor, and a graph of [protein standard] versus mean absorbance at 750nm.

2.15 PEPCK assay

PEPCK catalyses the decarboxylation and phosphorylation of oxaloacetate to form phosphenolpyruvate during gluconeogenesis. It also catalyses the reverse reaction (incorporation of carbon dioxide and dephosphorylation, with dGDP as the phosphate acceptor). The assay measures PEPCK activity by coupling oxaloacetate formation with its reduction by excess malate dehydrogenase to yield malate, with the equimolar consumption of NADH. The rate of the decrease in NADH concentration can be measured spectrophotometrically and is directly proportional to the activity of the PEPCK in a sample (Petrescu et al 1979).

Portions of liver from freshly killed rats were collected into Eppendorf tubes and 1.25ml ice-cold 250mM sucrose/ 5mM HEPES buffer pH 7.4 was added. The

samples were homogenised at 4°C and centrifuged at 12000g for 15 minutes at 4°C. The cytosolic supernatant was removed into ultracentrifuge tubes and spun for 45 minutes at 60000g at 4°C. The supernatant was removed into Eppendorf tubes and kept on ice. The appropriate amount of sample (20-60µl) was incubated at 30°C for 3 minutes in a reaction mixture containing: 50µl NADH (3.6mg/ml), 40µl phosphoenolpyruvate (10.4mg/ml), 50µl malate dehydrogenase (1.5iu, Roche Diagnostics Ltd, Lewes, UK) and made up to 1ml with buffer (50mM HEPES pH 6.5, 50mM sodium bicarbonate and 1mM manganese (II) chloride). The reactions were initiated in a spectrophotometer (set to 'Kinetic' mode) using 0.15mM dGDP. The drop in absorbance at 340nm was followed for three to seven minutes. Reaction mixtures lacking bicarbonate were used as negative controls. PEPCK activity was calculated from the rate of decrease in absorbance during the linear phase of the curve, and the molar absorptivity of NADH.

2.16 Northern blotting

2.16.1 RNA electrophoresis and capillary transfer

Total RNA was separated by electrophoresis on a 1.2% agarose formaldehyde denaturing gel. A 500ml gel was prepared by melting 6g of agarose in 440ml DEPC-treated water, adding 10ml formaldehyde and 10x MOPS buffer (50ml), and pouring into a gel mould with appropriately sized combs in place. The gel was allowed to set and was then pre-run in 1x MOPS buffer in a gel tank for 15 min at 80V. RNA was prepared for electrophoresis by aliquoting 20µg of RNA, adding DEPC-treated water to bring the total volume to 10µl, then adding deionised formamide (10µl), 40% formaldehyde (2.5µl) and 10x MOPS buffer (2.5µl) to give a total volume of 25µl. The sample was mixed and the RNA denatured by incubating at 65°C for 15min. Ethidium bromide was added to the loading buffer (1µl per 50µl loading buffer) and 2µl of this mix added to each sample of denatured RNA. The RNA was loaded into the wells on the gel and electrophoresed at 100V for 2 – 4 hours until the front band of the loading buffer was ¾ of the way down the gel. The gel was photographed under UV light (λ=254nm) with as little exposure as possible to minimise RNA damage. The gel was soaked in 20x SSC buffer for 15min before blotting onto a

nylon membrane (Zeta-Probe®). A wick of Whatman 3MM filter paper was placed over an upturned gel mould in a plastic tray containing 20x SSC buffer and the gel was placed on the top. A piece of nylon membrane cut to the same size as the gel and pre-wetted in 20x SSC was smoothed on top of the gel and this was covered with 3 layers of 3MM filter paper and approximately 5cm of paper towels. A plate of glass was placed on the top to secure the apparatus, and a weight balanced on the top. Capillary transfer was allowed to take place overnight at room temperature. The next day the membrane was washed in 20x SSC to remove any gel and the efficiency of transfer was checked by photographing the gel and the membrane under UV light (λ=254nm). The membrane was dried between two sheets of 3MM filter paper at 80°C for 2 hours, and the RNA cross-linked under UV light.

2.16.2 Hybridisation to ^{32}P-labelled cDNA

The nylon membrane was soaked in 20x SSC buffer for 5min, then placed in Hybaid hybridisation bottle containing 3ml 20% SDS and 6ml phosphate buffer that had been warmed to 55°C. To this was added denatured salmon sperm DNA (10mg/ml; 125µl). The membrane was prehybridised in a Hybaid hybridisation oven at 55°C for at least 2 hours. The ^{32}P-labelled cDNA probe (prepared as described in section 2.17.3) was added to the prehybridisation buffer, and the membrane hybridised with the probe overnight at 55°C. The probe was disposed of and the membrane was washed as follows: two washes in 1xSSC: 0.1%SDS at 55°C for 20min, and one wash in 0.3xSSC: 0.1%SDS at 55°C for 20min. The membrane was wrapped in cling-film and exposed to a Fujifilm imaging screen overnight for all probes (except U1, which was exposed for 10-15 minutes) and the level of hybridized probe quantified using a Fuji FLA2000 fluorescent image analyzer. The membranes were then rehybridised with U1 cDNA in the same way to control for RNA loading and transfer. The level of expression of the RNA of interest was then expressed as a ratio of the signal of the RNA of interest to the signal for U1. If results between groups of animals were to be compared all the samples were hybridised in the same hybridisation bottle with the same probe.

2.16.3 [^{32}P] Labelling of cDNA

DNA fragments were a kind gift from Dr D Livingstone. A random primed DNA labelling kit was used to label the DNA fragments (Roche Diagnostics Ltd UK). Approximately 25ng of DNA fragment was aliquoted into an Eppendorf, made up to 10µl with DEPC-treated water and denatured at 100°C for 10min. The Eppendorf was cooled on ice and briefly centrifuged to bring the contents to the bottom of the Eppendorf. Hexanucleotide primer mix (2µl), dATP, dTTP and dGTP (1µl of each), [α^{32}P]-dCTP (4µl) and Klenow (1µl) were added to give a total reaction volume of 20µl and the reaction incubated at 37°C for one hour. Unincorporated radioactivity was removed by passing the mixture over a NICK column (Pharmacia, St.Albans, UK). The NICK column was prepared by washing with 3ml TE then the reaction mixture was applied to the column. The column was washed with 400µl TE, the eluant discarded and the labelled DNA eluted from the column with a further 400µl TE. The activity of the probe was checked by mixing 2µl of probe with 1ml of scintillant and counting in a β-counter. The probe was used if the specific activity was greater than 70,000cpm/µl. The DNA probe was denatured before use by heating to 100°C for 5min.

2.17 11β-HSD 1 assay

In vivo, 11β-HSD1 is a reductase, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro,* reductase activity is labile and dehydrogenase activity predominates in tissue homogenates, so 11β-HSD1 activity was quantified by conversion of corticosterone to 11-dehydrocorticosterone.

Preliminary studies were carried out for each different tissue to optimise the protein concentration and incubation time to ensure that the percentage conversion was between 10 and 40% (ie not saturated).

2.17.1 Method 1

Aliquots of tissue homogenates at the appropriate concentration were incubated in duplicate at 37°C in C buffer containing [^{3}H]-corticosterone (10nM), NADP (400µM), for the times indicated in table 2.1. Blanks were prepared by incubating

[^{3}H]-corticosterone, NADP and C buffer with no tissue added. The reaction was stopped and steroids extracted by the addition of ethyl acetate. The organic phase was removed and evaporated. Extracts were resuspended in 35µl of a solution of corticosterone and 11-dehydrocorticosterone (0.5mg/ml in 100% ethanol) and analysed by thin layer chromatography using a mobile phase of 92% chloroform and 8% methanol.

2.17.2 Method 2

Aliquots of tissue homogenates at the appropriate concentration were incubated in duplicate at 37°C in Kreb's Ringer bicarbonate buffer containing 0.2% glucose, NADP (2 mM) and [^{3}H]-corticosterone (50 nM) in a total volume of 250µl, for the times indicated in table 2.1. Blanks were prepared by incubating [^{3}H]-corticosterone, NADP and buffer with no tissue added. After the incubation period the reaction was stopped by the addition of 10 volumes of ethyl acetate (all solvents used were HPLC grade). The organic phase was removed and evaporated under oxygen free nitrogen at 60°C. Extracts were re-suspended in mobile phase (20% methanol, 30% acetonitrile and 50% water) and stored at -20°C until analysis by HPLC. The percentage of [^{3}H]-B converted to [^{3}H]-A was corrected for apparent conversion in the blank samples, which was always <3%, and was used as an index of 11β-HSD 1 activity. Where results between different groups of animals were to be compared all the incubations were carried out concurrently, and all samples treated in the same way.

Tissue	Protein concentration	Incubation period	Method
Liver			
3 week/20 week	0.2mg/ml	15 min	1
24/72 hour	50μg/ml	1 hour	2
Subcutaneous fat			
3 week	0.5mg/ml	3 hours	1
20 week	0.4mg/ml	6 hours	1
24/72 hour	500μg/ml	2 hours	2
Omental fat			
3 week/20 week	0.5mg/ml	3 hours	1
24/72 hour	250μg/ml	2 hours	2

Table 2.1 Incubation times, protein concentrations and methods used for 11β-HSD 1 assay on tissues from Wistar rats on high fat or control diets. 3 weeks, 20 weeks and 24/72 hours refer to length of time on high fat and control diets as described in chapter five.

2.18 5β- Reductase assay

100mg of liver was homogenised in sucrose buffer (0.25M, pH7.5), HEPES (10mM) and dithiothreitol (1mM). Cytosolic and microsomal sub-fractions were separated by differential centrifugation. Briefly, homogenised liver samples were centrifuged (1000g 4°C, 10 min) and the supernatant removed and centrifuged (34,000g, 4°C, 30 min). The resulting supernatant was further centrifuged (124,000g, 4°C, 60 min), yielding cytosolic supernatant for analysis. The protein concentration was determined colorimetrically. Cytosolic preparations (400μg/ml protein) were incubated in duplicate at 37°C in potassium phosphate buffer 0.1M, pH 7.5 containing glucose-6-phosphate (5mM), glucose-6-phosphate dehydrogenase (0.1 units/ml), NADPH (2mM), [^{3}H] corticosterone (50nM) and unlabelled corticosterone

(9.95μM). After 2 hours, steroids were extracted with ethyl acetate, dried down and resuspended in mobile phase (water:acetonitrile:methanol; 60:10:30). Steroids were separated by HPLC, and their concentrations quantified by on-line scintillation counting. 5β-reductase activity was expressed as % conversion of [^{3}H] corticosterone to [^{3}H] 5β-tetrahydrocorticosterone (5β-THB).

2.19 Real-Time PCR

Real-time polymerase chain reaction (PCR) was performed to quantify mRNA for 11β-HSD 1, GR and 5α-reductase in liver and fat.

2.19.1 Preparation of cDNA

RNA was extracted from tissues as described in section 2.13. cDNA was synthesised from 0.5μg RNA samples using the Promega Reverse Transcription System (Promega, Southampton, Hants, UK) in a reaction mixture containing 25mM $MgCl_2$, 10x reverse transcription buffer, 10mM each of dATP, dCTP, dGTP and dTTP, 20U RNasin, 0.5μg Oligo$(dT)_{15}$ primers and 15U AMV-reverse transcriptase made up to 50μl in DEPC-treated water. Samples were incubated at 42°C for 50 min followed by 5 min at 99°C then 5 min on ice to inactivate enzymes and prevent binding to DNA.

Negative control reactions for each RNA sample were performed in parallel (made up as above but in the absence of AMV-reverse transcriptase) in order to exclude genomic DNA contamination. A further negative control reaction containing water instead of RNA was performed to determine RNA contamination of the Reverse Transcriptase System reagents.

PCR amplification was performed on a third to one half of the cDNA samples to confirm successful reverse transcription as described in section 2.20.2, using Glyceraldehyde 3-phosphate dehydrogenase primers (table 2.2). cDNA was diluted 1:4, aliquotted and stored at -20°C until use.

PCR amplification of samples using the Real Time PCR primers and probes for 11β-HSD 1, GR and cyclophilin showed amplification of products of the appropriate size.

2.19.2 PCR reactions

5µl of cDNA template was used in each PCR reaction containing 2.5µl of 10x Thermophilic DNA polymerase Reaction Buffer containing 25mM $MgCl_2$ and made up to a final volume of 25µl with DEPC-treated water. A second mixture containing 2.5µl of 10x Thermophilic DNA polymerase Reaction Buffer containing 25mM $MgCl_2$, 2.5mM each of dATP, dCTP, dGTP and dTTP, 15pmol upstream primer, and 15pmol downstream primer was made up to 25µl in DEPC-treated water. 0.25µl of *Taq* polymerase was added to each reaction tube just prior to starting the required PCR programme. A negative control reaction containing DEPC-treated water rather than cDNA was performed in parallel to determine contamination of PCR reagents.

PCRs were performed on an Eppendorf Mastercycler Gradient with a heated lid (set to 110°C). Samples were heated to 95°C for 3min for initial denaturation, placed directly on ice for 1min before the addition of the second mixture. All samples then underwent 35 cycles of PCR amplification (denaturation at 94°C for 1min, primer annealing at primer-specific temperature for 1 min and elongation at 72°C for 2 min). Upon completion of the PCR programme, samples were incubated at 72°C for a further 10 min to ensure elongation of products to full length and cooled to 4°C prior to gel electrophoresis.

RT-PCR products were analysed by electrophoresis on a 1.0% agarose gel using 10µl 1Kb DNA ladder containing fragments ranging from 75-12,000 bases to allow determination of product size.

Mouse GAPDH	Sequence (5'-3')	Accession number	Amplicon length	Tm (°C)	cDNA position
FP RP	gtcggtgtgaacggatttggccgt catggcctacatggcctccaagg	M32599	1001	56	51-79 1021-1043

Table 2.2 PCR primer sequences for GAPDH.

FP= forward primer, RP= reverse primer. Annealing temperature = 56°C; 35 cycles

Gene	Sequence (5'-3')	Accession number	Amplicon length	cDNA position
Cyclophilin FP RP Probe	cccaccgtgttcttcgacat gaaagttttctgctgtctttggaact caagggctcgccatcagccgt	M19533	99	52-71 126-151 73-93
GR FP RP Probe	gggtactcaagccctggaatg cccgtaatgacatcctgaagct ccacgggaccacctcccaagc	NM012576	126	1302-1322 1363-1383 1405-1427
11β-HSD 1 FP RP Probe	tcatagacacagaaacagctttgaaa ctccagggcgcattcct ctgggataatcttgagtcaagctgctccc	NM017080	83	725-750 758-786 791-807
5α-R 1 FP RP Probe	ctgtttcctgacaggctttgc gcctcccctgggtatcttgt cagaccacatcctgaggaatctgagaa aacc	J05035	100	444-464 545-564 500-530

Table 2.3 Real Time PCR primers and probe sequences, amplicon lengths and cDNA position. 5α-R 1 = 5α-reductase type 1. Annealing temperatures: cyclophilin 59°C, 11β-HSD 1 52°C, 5α-reductase 59°C. FP= forward primer, RP= reverse primer.

2.19.3 Real-time PCR probes and Primers

Exon-spanning probes and primers were designed using Primer Express software (Applied Biosystems, Warrington, UK) and are detailed in table 2.3. Cyclophilin was chosen as the endogenous control.

GR, 11β-HSD 1 and 5α-reductase probes were labelled with the fluorescent dye FAM, and cyclophilin with the fluorescent dye VIC.

Reactions were carried out on 384 well plates using a final volume of 10μl per well. A master TaqMan mix was made, consisting of (per well) 300nM forward and reverse primers, 200nM probe, 2.5μl cDNA, 6.86μl TaqMan Universal PCR Master Mix (Applied Biosystems, Applera, Warrington, UK). Samples were run in triplicate where possible, or otherwise in duplicate. Four wells per gene of interest were used for a –RT sample generated during the RT-PCR, and two wells for a 'no template control' reaction using nuclease-free water in place of cDNA.

A preliminary experiment demonstrated that 300nM both forward and reverse primers and 200nM probe gave good amplification plots.

cDNA from the tissue of interest was pooled prior to dilution to use for the generation of a standard curve. This stock cDNA was used in dilutions of 1, 1:2, 1:4, 1:8, 1:16, 1:32 and run in triplicate to generate a standard curve for relative quantification of the target mRNA in samples.

Plates were analysed on a TaqMan™ ABI Prism 7900 Sequence Detector™. Cycling parameters: 50°C for 2 mins, 95° for 10 mins, 40 cycles of 95°C for 15 secs, 60°C for 1 min. Data acquisition was processed with Sequence Detector 1.6.3 software.

2.20 Diet constituents

Diets were supplied by Research Diets, New Brunswick, New Jersey

	Control diet D01072401		**High fat diet D12451**	
	(g)	kcal %	(g)	kcal %
Composition				
Protein	19.2	20	23.7	20
Carbohydrate	67.3	70	41.4	35
Fat	4.3	10	23.6	45
Total	90.8	100	88.7	100
Kcal/g	3.85		4.73	
Ingredients	***(g)***	***kcal***	***(g)***	***kcal***
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn starch	452	1809	72.8	291
Maltodextrin 10	75	300	100	400
Sucrose	172.8	691	172.8	691
Cellulose	50	0	50	0
Soybean oil	25	225	25	225
Lard	20	180	177.5	1598
Mineral mix S10026	10	0	10	0
DiCalcium Phos	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	1055	4057	858	4057

2.21 Statistics

All values are expressed as mean ± standard error. Data were statistically analysed by Student's *t* tests or Analysis of Variance followed by post-hoc LSD testing.

Chapter Three - Intergenerational effects of glucocorticoid programming in the rat

3.1 Introduction

As discussed in the introduction, many epidemiological studies have demonstrated a link between low birth weight and a number of cardiovascular risk factors (Barker 1998). Evidence from human studies suggests that this phenomenon may not be limited to the first generation and that intergenerational effects may operate, resulting in the persistence of programming effects in subsequent generations. Epidemiological studies in humans suggest that there may be intergenerational effects on birth weight (Ounsted & Ounsted 1968, Klebanoff et al 1984, Carr-Hill et al 1987, Klebanoff et al 1989, Emanuel et al 1992, Klebanoff et al 1997, Emanuel et al 1999), cardiovascular risk factors (Davey Smith et al 1997, Walker et al 1998, Lawlor et al 2002, Kuznetsova et al 2003) and type 2 diabetes (Alcolado & Alcolado 1991, Klein et al 1996, Karter et al 1999, Dabelea et al 2000).

Intergenerational effects have been explored in animal models; in utero exposure to a low protein diet has intergenerational effects on birth weight and the endocrine pancreas (Stewart et al 1975, Stewart et al 1980, Hoet & Hanson 1999, Martin et al 2000); postnatal dietary manipulations have been shown to have second generation effects on glucose homeostasis (Laychock et al 1995, Vadlamudi et al 1995, Patel et al 2001, Srinivasan et al 2003); and postnatal environmental manipulations may have intergenerational effects on the HPA axis (Francis et al 1999).

One proposed mechanism for fetal programming is that of overexposure of the fetus to glucocorticoids (Edwards et al 1993). Rats exposed to dexamethasone (dex) during the last third of pregnancy are of low birth weight and develop hypertension (Benediktsson et al 1993), glucose intolerance (Nyirenda et al 1998) and HPA axis activation (Levitt et al 1996, Welberg et al 2001) in adulthood. The glucose intolerance is thought, at least in part, to be mediated by increased activity of the

hepatic gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Nyirenda et al 1998).

Using this model of programming, we have explored intergenerational effects on birth weight, hepatic PEPCK, glucose tolerance and blood pressure.

3.2 Methods

Female Wistar rats weighing around 250g were timed-mated with Wistar males and then caged separately throughout gestation. Pregnant females were injected subcutaneously with a solution of dexamethasone in 0.9% saline containing 4% ethanol (100μg per kg body weight), or with an equivalent volume of vehicle (1ml/kg), at the same time each morning between days 15 and 21 of pregnancy inclusive (n=8 per group).

Litters (F1 dex and F1 veh) were weighed at birth and culled to eight pups (n=8 dex litters, 7 veh litters). Pups were weaned at three weeks of age and housed with littermates or with same aged pups from the same treatment group. F1 females weighing around 230-250g were timed-mated with F1 males from different litters of the same prenatal treatment group (n=10 per group). Females were caged separately during pregnancy and not manipulated in any way. Pups (F2 dex and F2 veh) were weighed at birth and culled to eight (n=9 litters for both groups). Pups were weaned at 3 weeks.

F2 females were timed-mated with unrelated males from the same F2 group (n= 10 per group). 10 litters were obtained for the dex group (F3 dex) and 9 for the veh group (F3 veh). Pups were weighed at birth and each litter culled to eight.

All offspring were weighed at weaning and monthly thereafter. In all cases, experimental cohorts included males and females selected randomly from as many litters as possible.

At 12 weeks (F1 and F3) or five weeks (F2) of age, male animals (n=8-10) from each group were culled by cervical dislocation. Portions of liver were collected into Eppendorfs and placed on wet ice for PEPCK analysis. The elevation in hepatic PEPCK activity in dex animals has previously been shown to be stable between 21 days and 8 months of age (Nyirenda et al 1998).

Glucose tolerance tests were conducted on F1 males and females at 6 months of age, on F2 animals at 4 and 6 months of age, and on F3 animals at 6 and 15 months. Plasma glucose was determined by the enzymatic (hexokinase) method, and plasma insulin by ELISA.

Corticosterone levels were measured by radioimmunoassay on tail nick plasma samples taken between 0800 and 0900 hours following daily handling of the animals for 10 days to 2 weeks.

Blood pressure was measured in animals at rest at the same time each day following carotid cannulation by Dr Patrick Hadoke, Endocrinology Unit, University of Edinburgh.

Data are expressed as mean ± SEM unless otherwise stated. Data were analysed using unpaired *t* tests, 2-way or repeated measures ANOVA (glucose tolerance tests, longitudinal body weight) where appropriate. Significance was set at P of 0.05 or less.

3.3 Results

3.3.1 F1 cohort

The F1 offspring of dex-treated mothers (F1 dex, n=74) were significantly lighter than the F1 offspring of veh-treated mothers (F1 veh, n=81) (Figure 3.1), in agreement with previous studies (Levitt et al 1996, Nyirenda et al 1998, Cleasby et al 2003a). There were no changes in gestation length, litter number or proportions of male and female offspring (Table 3.1). Females receiving dexamethasone during the last week of pregnancy gained less weight than those receiving vehicle injections, consistent with previous reports (Table 3.1). Hepatic PEPCK activity tended to be elevated in the liver of male F1 dex offspring (Figure 3.2). Although this result did not reach significance, mean values are consistent with those from previous studies (Nyirenda et al 1998).

Growth trajectories for male and female animals are shown in figure 3.3. Although F1 dex males achieved catch-up growth by weaning at 21 days and thereafter maintained equivalent weights to their F1 veh counterparts, F1 dex females never achieved full catch-up growth. Thus, F1 dex females were lighter than F1 veh females at the time of mating.

There were no differences between F1 dex and F1 veh animals in glucose tolerance (Figures 3.4 and 3.5), basal corticosterone levels (Table 3.1) or blood pressure (Table 3.1). Basal corticosterone levels in females were significantly higher than those in males, in accordance with previous studies (Atkinson & Waddell 1997).

	F1 dex	F1 veh	P value
Gestation length (days)	20.8 ± 0.3	20.9 ± 0.1	0.73
Litter number	11.5 ± 0.8	12.7 ± 0.7	0.28
Maternal weight gain (g)	25.6 ± 3.2	57.2 ± 3.7	<0.001
Basal plasma corticosterone (nmol/l) at 9 months			
Males	30 ± 3	25 ± 3	0.37
Females	517 ± 100	661 ± 150	0.4
Mean blood pressure (mmHg) at 14 months			
Males	132	132 ± 2	-
Females	115 ± 6	115 ± 7	0.94

Table 3.1 F1 cohort data for 8 dex litters and 7 veh litters.

Data are mean ± SEM and were analysed by Student's *t* test.

Corticosterone levels were measured in 6 veh males and 9 dex males and in 8 females per group.

Blood pressure was measured in 5 F1 veh and 6 F1 dex females, but because of technical problems, only in four F1 veh and two F1 dex males.

3.3.2 F2 cohort

Birth weight was reduced in the F2 dex offspring when compared with the F2 veh offspring, although both groups were heavier than their F1 counterparts (Figure 3.1). Although the F1 dex mothers were lighter than the F1 veh mothers at gestational day E0 (Table 3.2), there was no difference in maternal weight gain during pregnancy (Table 3.2), in litter number, or in the proportions of male and female offspring (Table 3.2). Hepatic PEPCK activity was elevated in the livers of male F2 dex animals (Figure 3.2). F2 dex males and females both achieved catch-up growth by weaning (Figure 3.3).

In previous F1 cohorts, glucose intolerance has not been noted before 6 months of age (Dr Moffat Nyirenda, unpublished observations). However, in view of the marked difference in PEPCK activity in this F2 cohort, glucose tolerance tests were performed at 4 months as well as 6 months on F2 males and females. At 4 months there were no differences in plasma insulin in the males (Figure 3.4) or in glucose or insulin in the females during glucose tolerance testing (Figure 3.5). However, the F2 dex males had higher glucose values during the glucose tolerance test (Figure 3.4). At 6 months, F2 dex males demonstrated higher basal insulin levels and an elevated basal insulin:glucose ratio (Table 3.2). Although F2 dex females did not demonstrate overt glucose intolerance or insulin resistance at 6 months, there was a trend towards an elevated basal insulin: glucose ratio (Table 3.2).

There were no differences in basal corticosterone levels (Table 3.2) in males or females. F2 dex males had significantly elevated mean and systolic blood pressures when measured by carotid cannulation at 13 months of age (Table 3.2). In contrast, there was no difference in blood pressure in F2 females when measured at 17 months (Table 3.2).

	F2 dex	F2 veh	P value
Litter number	11.6 ± 0.9	12 ± 0.6	0.68
Maternal weight at E0 (g)	241.8 ± 4.3	259.4 ± 3.2	0.005
Maternal weight gain (g)	100.4 ± 6.4	104.5 ± 5.2	0.63
Plasma corticosterone (nmol/l) at 12 months			
Males	180 ± 37	202 ± 31	0.65
Females	274 ± 96	379 ± 81	0.42
Mean blood pressure (mmHg)			
Males (13 months)	137 ± 3	128 ± 3	0.037
Females (17 months)	131 ± 3	125 ± 4	0.21
Systolic blood pressure (mmHg)			
Males (13 months)	150 ± 2	141 ± 3	0.03
Females (17 months)	142 ± 3	135 ± 4	0.24
Basal insulin (ng/ml) at 6 months			
Males	1.08 ± 0.05	0.92 ± 0.04	0.036
Females	0.25 ± 0.02	0.21 ± 0.02	0.18
Basal insulin:glucose ratio (6 months)			
Males	0.20 ± 0.09	0.17 ± 0.08	0.009
Females	0.050 ± 0.005	0.036 ± 0.004	0.057

Table 3.2 F2 data.

Data are for 9 litters per group. E0 = day when vaginal plug noted. Basal corticosterone levels, n=8 per group. Mean and systolic blood pressures measured by carotid cannulation (n=9 F2 veh males and 10 F2 dex males; n=8 F2 dex females and 6 F2 veh females). Basal insulin and insulin: glucose ratios for 9 F2 dex males and 8 F2 veh males; 10 F2 females per group.

Data are mean ± SEM and were analysed by Student's *t* test.

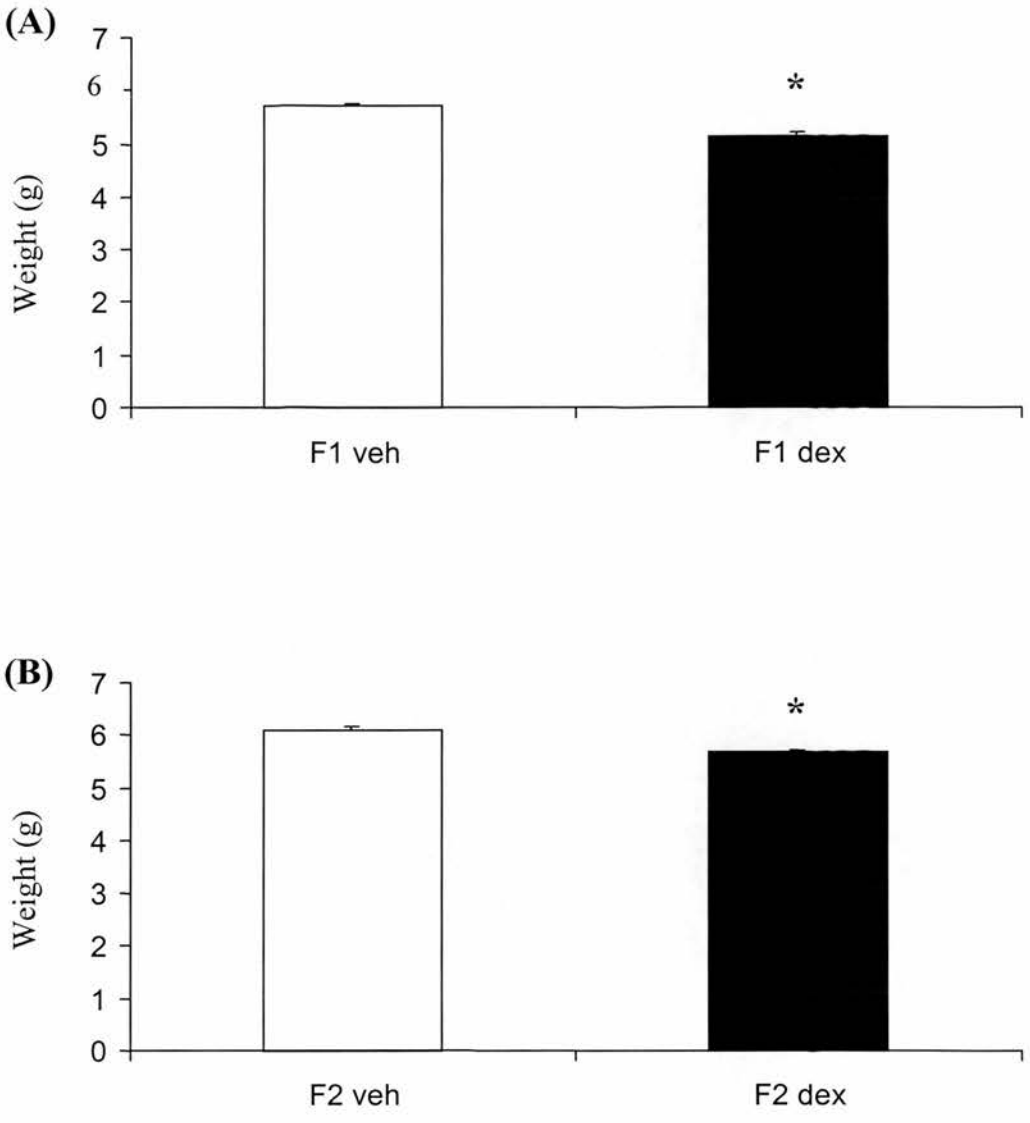


Figure 3.1 Birth weights in F1 and F2 animals.

Birth weights in F1 (panel A n= 81 veh, n=74 dex) and F2 (panel B n=108 veh, n=101 dex) animals.

Data were analysed by Student's *t* test * denotes $p<0.05$.

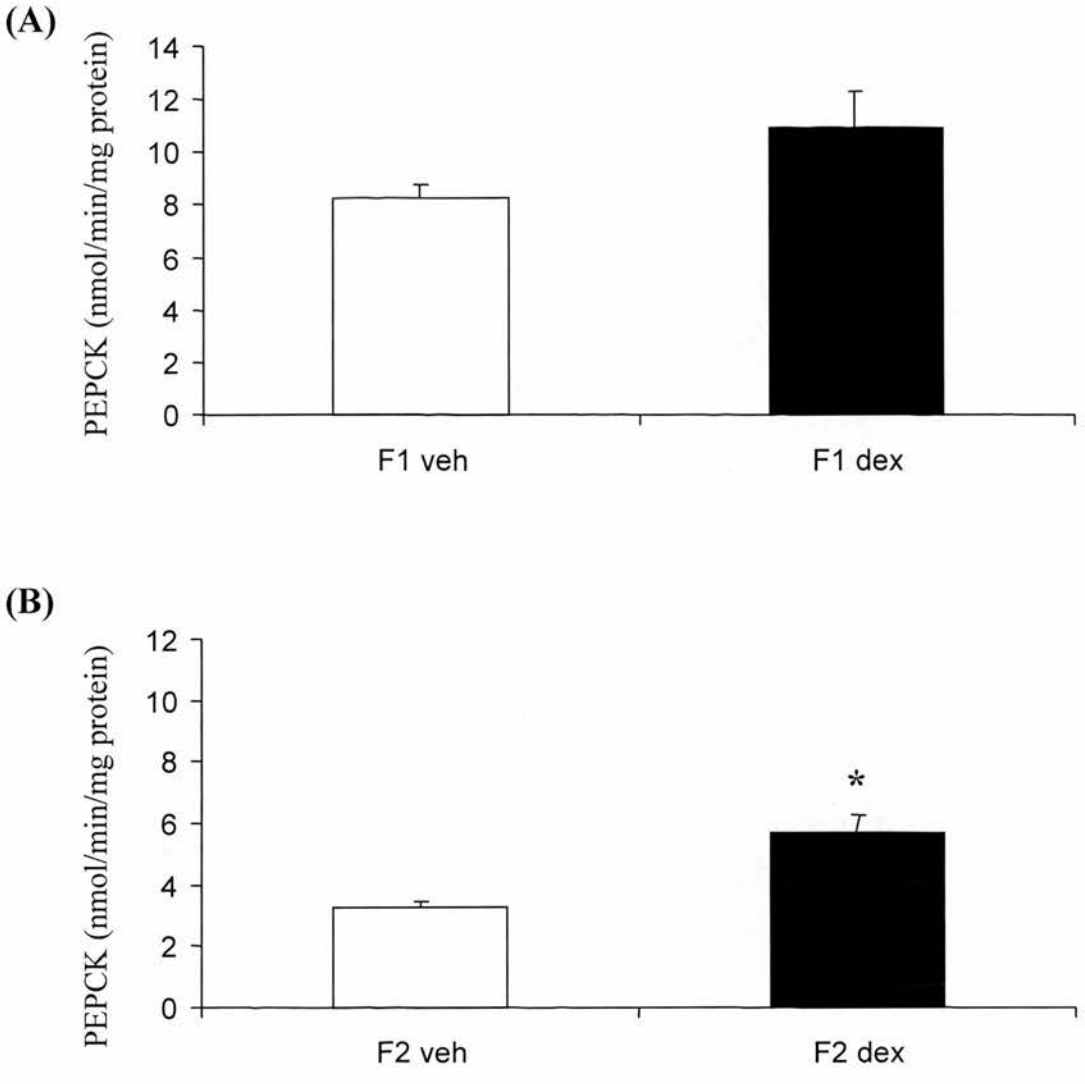


Figure 3.2 Hepatic PEPCK activity in F1 and F2 males.

PEPCK activity in F1 males at 12 weeks of age (panel A, p=0.097) and F2 males at 5 weeks of age (Panel B), n=8 per group.

Data were analysed by Student's *t* test * denotes $p<0.05$.

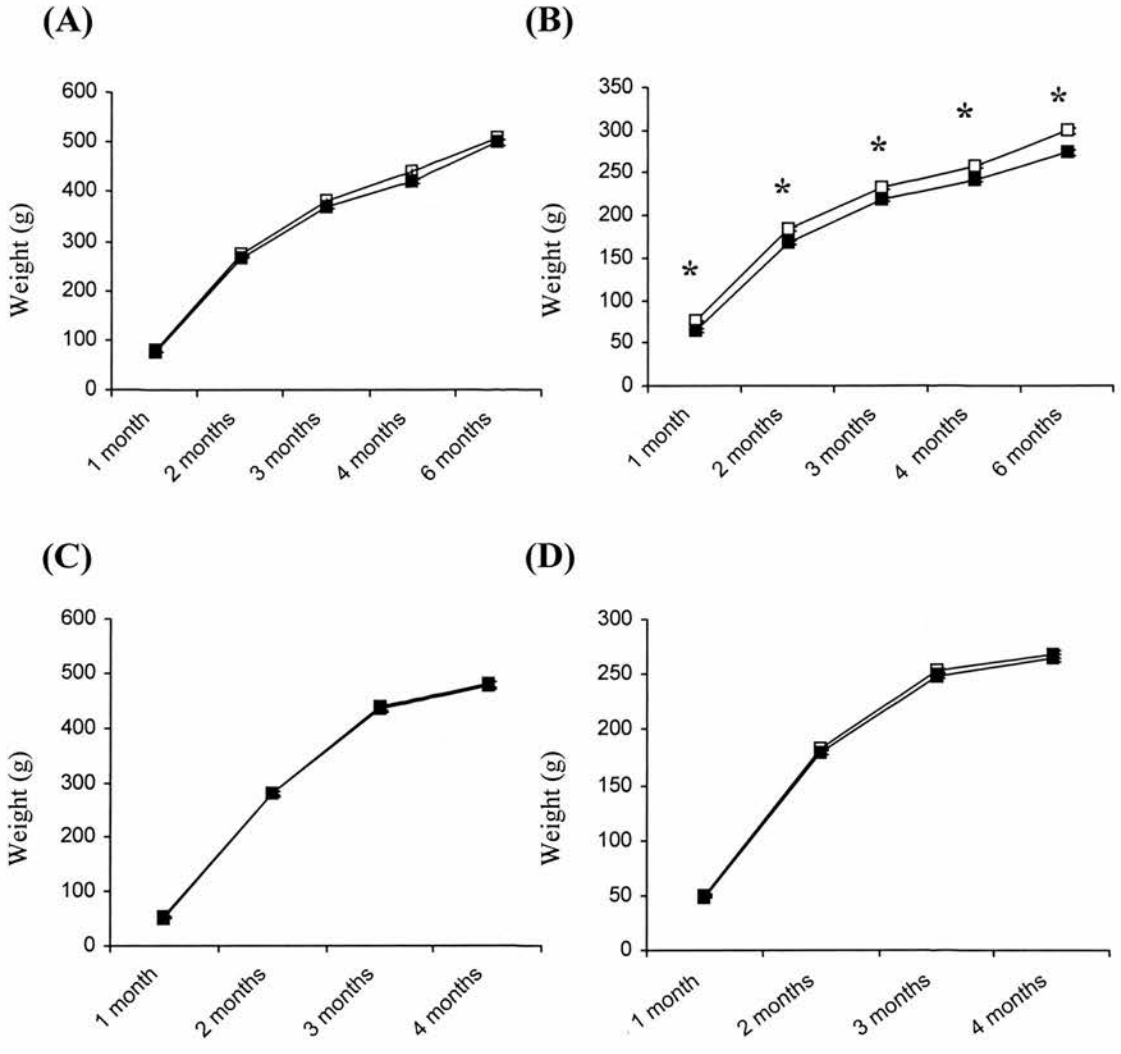


Figure 3.3 Weight trajectories F1 and F2 cohort.

Weight gain in F1 males (panel A, n=20-30 per group) and females (panel B, n=10-13 per group).

Continuing weights in F2 males (panel C, n= 68 per group at 1 month, 29 at months 2-3 and 24 at 4 months) and females (panel D, n=30 per group at 1-3 months and 20 at 4 months). Data were analysed by ANOVA, * indicates $p<0.05$).

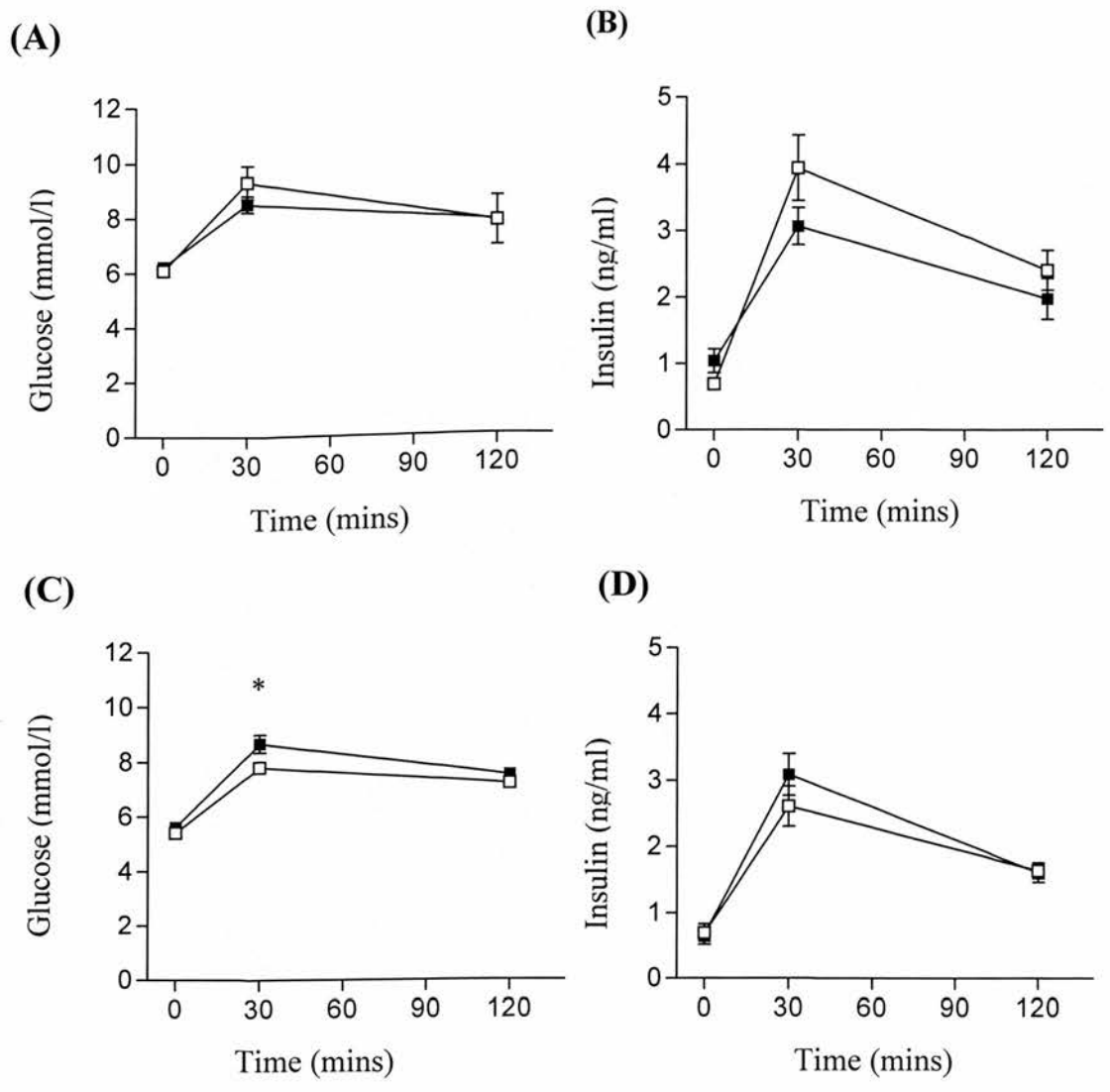


Figure 3.4 Glucose and insulin in F1 and F2 males on glucose tolerance testing.

Plasma glucose (panel A) and insulin (panel B) in F1 males at 6 months (n=10 per group).

Plasma glucose (panel C) and insulin (panel D) in F2 males at 4 months (n=10 per group).

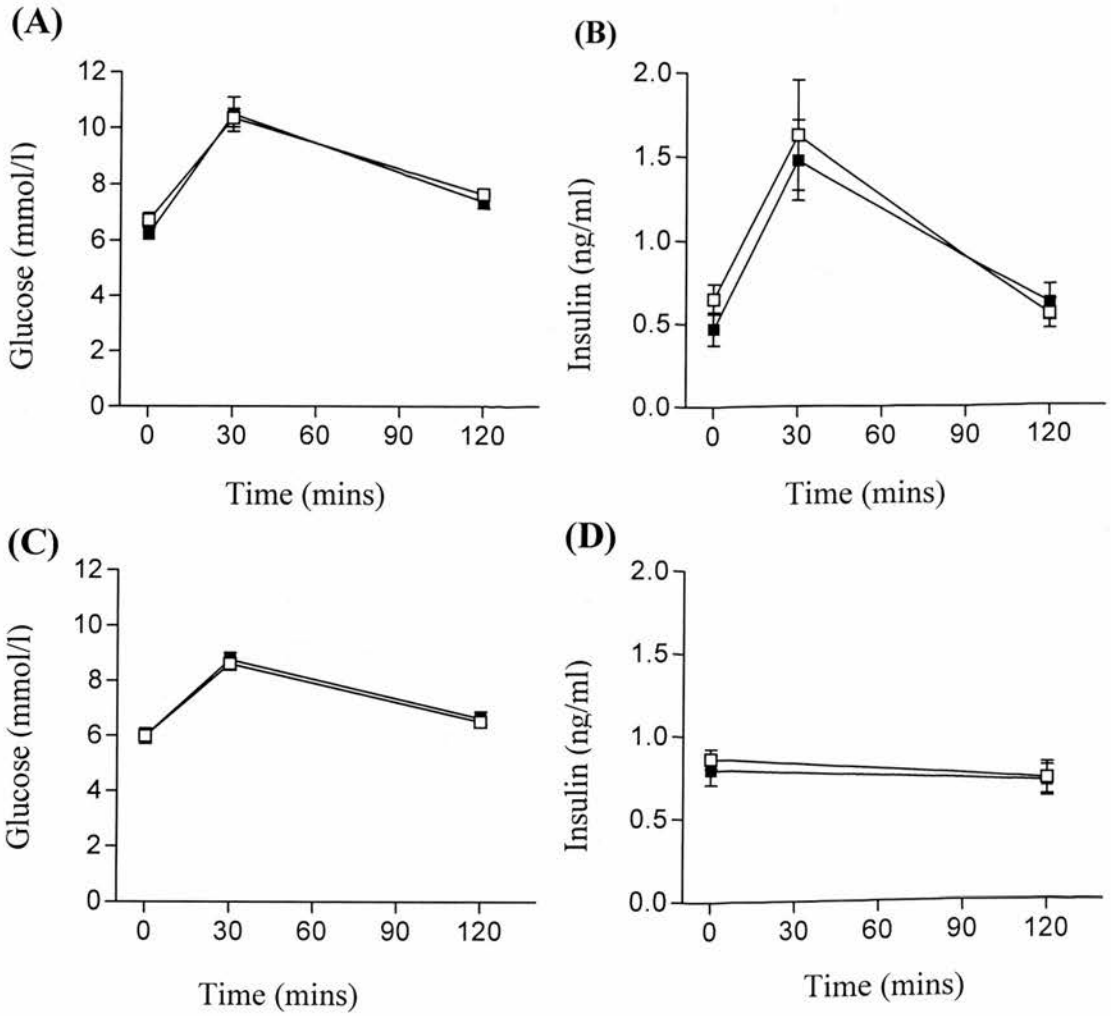
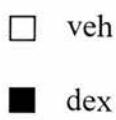


Figure 3.5 Glucose and insulin in F1 and F2 females on glucose tolerance testing. Plasma glucose (panel A) and insulin (panel B) in F1 females at 6 months (n=10 per group). Plasma glucose (panel C) and insulin (panel D) in F2 females at 4 months (n=10 per group).

Analysis by ANOVA revealed no differences between groups in either cohort.



3.3.3 F3 cohort

There were no differences in birth weight between F3 dex and F3 veh litters (Table 3.3) or in hepatic PEPCK activity in male offspring (Table 3.3). There were no differences between maternal weights at gestation E0, litter number, gestation length, proportions of male and female offspring (Table 3.3) or in the postnatal growth trajectories of F3 dex and F3 veh males and females.

Glucose tolerance testing was performed in males and females at 6 and 15 months of age; no differences between dex and veh animals were found in glucose values for males or females at either time point (Figure 3.6).

	F3 dex	F3 veh	P value
Birth weight (g)	5.90 ± 0.05	5.80 ± 0.06	0.22
Gestation length (days)	21.6 ± 0.2	21.8 ± 0.2	0.61
Litter number	10.7 ± 0.7	12.0 ± 0.5	0.17
Maternal weight at E0 (g)	268 ± 7.7	267 ± 4.7	0.93
Maternal weight gain (g)	117.3 ± 5.8	118.2 ± 2.7	0.89
PEPCK activity (nmol/min/mg protein)	9.97 ± 1.14	10.61 ± 0.61	0.62

Table 3.3 F3 cohort data

Data are for 10 F3 dex and 9 F3 veh litters. E0 = day when vaginal plug noted. Birth weights are for 108 F3 veh and 107 F3 dex animals. PEPCK activity was measured in 8 F3 veh and 7 F3 dex animals at 12 weeks of age.

Data are mean ± SEM and were analysed by Student's *t* test.

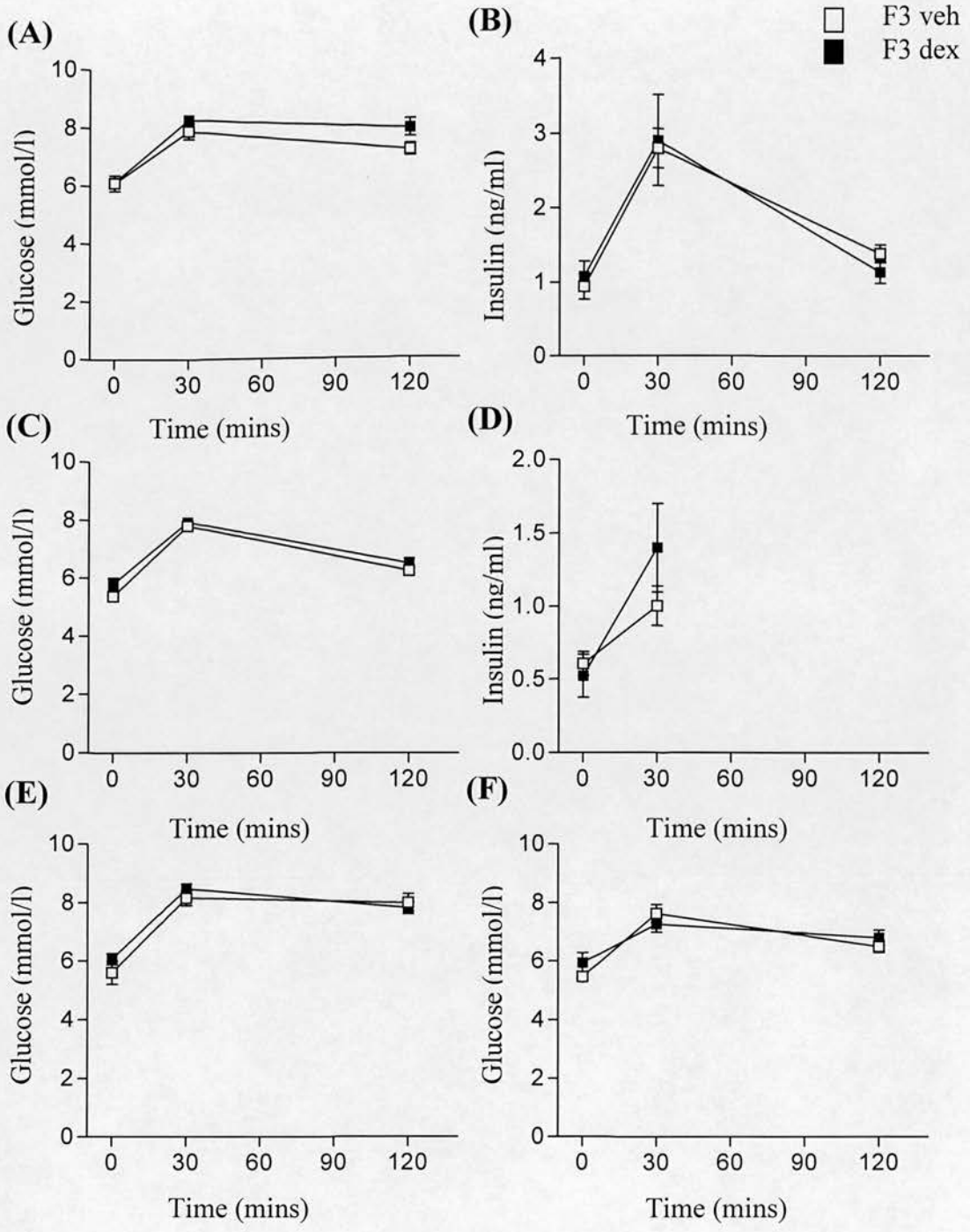


Figure 3.6 Glucose tolerance tests in F3 males and females at 6 and 15 months. Plasma glucose (panel A) and insulin (panel B) in F3 males at 6 months. Plasma glucose (panel C) and insulin (panel D) in F3 females at 6 months. Plasma glucose in F3 males (panel E) and females (panel F) at 15 months.

Analysis of data by ANOVA revealed no significant differences between groups at either time point (n=8 per group).

3.4 Discussion

These data demonstrate intergenerational effects in the dexamethasone-programmed rat. Rats exposed to dexamethasone *in utero* have reduced birth weight, elevated hepatic PEPCK and abnormal glucose tolerance (Nyirenda et al 1998) and we show for the first time that the offspring of these rats, without further manipulation also exhibit these features. These effects are no longer evident in a third generation.

Consistent with previous reports, birth weight was reduced in rats exposed to dexamethasone *in utero* during the last week of pregnancy (Levitt et al 1996, Nyirenda et al 1998, Welberg et al 2001, Cleasby et al 2003a). Birth weight was reduced by 9% in the F1 dex animals, in agreement with previous studies which have reported reductions in birth weight of between 6 and 11% (Levitt et al 1996, Nyirenda et al 1998, Welberg et al 2001). Maternal weight gain was also reduced in the dexamethasone treated animals, but no effects were seen on gestation length, litter size, or offspring viability in the F1 generation.

In the F1 generation, there was a trend for hepatic PEPCK activity to be elevated. Treatment with dexamethasone during the third week of pregnancy has been shown to induce permanent overexpression of hepatic PEPCK in the F1 offspring, with no significant effect on other gluconeogenic enzymes (Nyirenda et al 1998); PEPCK expression is altered from day 5 of postnatal life, and remains elevated at 8 months (Nyirenda et al 1998). PEPCK is the rate-limiting enzyme of gluconeogenesis (Pilkis & Granner 1992), and transgenic mice with overexpression of hepatic PEPCK have impaired glucose tolerance (Valera et al 1994). In addition, over-expression of PEPCK in a rat hepatoma cell line impairs suppression of gluconeogenesis by insulin (Rosella et al 1993). It has therefore been proposed that the increase in PEPCK in programmed animals may increase hepatic gluconeogenesis, contributing to the glucose intolerance reported in these animals (Nyirenda et al 1998). The mechanism behind this programmed increase in PEPCK is unclear, but may be mediated by the reported increase in hepatic glucocorticoid receptor number (Nyirenda et al 1998), in association with normal or increased plasma glucocorticoid levels.

The postnatal growth trajectories of the F1 dex animals revealed a sexually dimorphic pattern. The F1 dex males achieved catch-up growth by weaning, whereas the F1 dex females remained smaller throughout the period of follow-up (1 year). Previous reports have noted different growth patterns in rats exposed to dexamethasone during the last week of gestation. Catch-up growth has been reported to be complete (Nyirenda et al 1998, Welberg et al 2001) or incomplete (Cleasby et al 2003a) in different groups of animals receiving the same antenatal treatments. Postnatal growth has been proposed to be an important factor modifying the risk of subsequent disease associated with low birth weight. In some human studies, slow growth during the first year of life has been associated with an increased risk of cardiovascular disease in later life (Eriksson et al 2001), although a recent study has suggested that rapid growth during the first two weeks of life may also be associated with an increased risk of cardiovascular disease (Singhal et al 2003). Increased growth in the first two years of life is associated with an increased risk of obesity in childhood, which may be a predisposing factor to obesity in adulthood (Ong et al 2000). Furthermore, accelerated growth during childhood, after the first year, increases the risk of hypertension, type 2 diabetes and coronary heart disease (Eriksson et al 1999, Eriksson et al 2000, Forsen et al 2000, Eriksson et al 2001, Eriksson et al 2002, Law et al 2002, Eriksson et al 2003). Factors governing postnatal catch-up growth and the mechanisms by which it modifies disease risk remain unclear, although potential mechanisms include programming of appetite and/or obesity (reviewed in Breier et al 2001), or modification of postnatal growth and metabolism by genetic factors (reviewed in Ong & Dunger 2002).

The role of catch-up growth in modifying the phenotype of the glucocorticoid-programmed rat is unclear. In addition to studies demonstrating the programming effects of *in utero* glucocorticoid overexposure in the last week of gestation, rats exposed to excess glucocorticoid throughout gestation, either by maternal administration of dexamethasone or carbenoxolone (which inhibits placental 11β-HSD), demonstrate reduced birth weights (Benediktsson et al 1993, Lindsay et al 1996a, Lindsay et al 1996b, Nyirenda et al 1998, Welberg et al 2000, Welberg et al 2001) but different patterns of postnatal growth (Lindsay et al 1996b, Nyirenda et al 1998, Welberg et al 2000, Welberg et al 2001). Nevertheless, programmed effects on

glucose tolerance and behaviour have been reported in groups of animals in the different programming paradigms, with or without postnatal catch-up growth (Lindsay et al 1996b, Nyirenda et al 1998, Welberg et al 2000, Welberg et al 2001, Cleasby et al 2003a).

No differences were noted in glucose tolerance or basal corticosterone levels in F1 animals. Some, but not all, studies have noted an increase in basal corticosterone level in dexamethasone-programmed animals (Levitt et al 1996, Nyirenda et al 1998). No difference was detectable in blood pressure in F1 males or females, although technical difficulties meant that blood pressure was only measured in a small number of F1 males. The lack of an effect on glucose homeostasis, and on plasma corticosterone levels, may indicate that the programming effect in this F1 cohort was relatively mild. Additionally, experience in our laboratory over some years suggests that external stressors, such as building work near to the animal unit, or ultrasonic noise from lighting systems can result in cohort differences in values in the placebo treated groups.

Birth weight was reduced in the F2 dex offspring and in addition, hepatic PEPCK was significantly increased. This marked effect in the F2 generation appears to have occurred despite a mild programming phenotype in the F1 dex parents.

In contrast to the lack of any detectable effect on glucose homeostasis in the F1 generation, at four months of age the F2 dex males demonstrated higher glucose levels at 30 minutes following a glucose load. Previous studies in F1 animals have not been able to show evidence of glucose intolerance in animals exposed to dexamethasone *in utero* until 6 months of age (Dr M Nyirenda, unpublished data). By six months, the F2 dex males had elevated basal plasma insulin levels and an elevated fasting insulin:glucose ratio, indicating insulin resistance. In the females, the effect was less clear, although there was a trend for an elevation in fasting insulin:glucose ratio at 6 months. The observed glucose intolerance in the F2 animals may be explained in part by programmed hepatic PEPCK overexpression leading to increased gluconeogenesis. A recent study, while demonstrating reduced glycogen

storage in the muscle of F1 dex animals, did not find evidence of reduced peripheral glucose uptake and found no difference in protein levels of the glucose transporter GLUT-4 in the muscle of programmed animals (Cleasby et al 2003a). Glucose uptake into adipose tissue was similarly unchanged (Cleasby et al 2003a). These results suggest that enhanced hepatic glucose output may be the main factor in determining the programmed hyperglycaemia observed in the programmed animals. Consistent with this observation, the F2 dex animals in this study showed evidence of glucose intolerance in addition to a marked elevation in PEPCK activity, in contrast to the F1 dex animals, which showed normal glucose tolerance (at least in a glucose tolerance test) and a trend for elevated PEPCK activity.

There were no detectable programming effects in the third generation, in which there were no differences in birth weight and subsequent growth patterns, hepatic PEPCK activity or glucose tolerance between F3 dex and F3 veh animals.

There are a number of possible explanations for this intergenerational phenomenon, as discussed in chapter one, including programmed effects in the mother, resulting in the exposure of the fetus to an adverse environment in utero, and thus perpetuating the programming effects in subsequent generations. Maternal size has been shown to be an important determinant of fetal size in early studies using shire horses and Shetland ponies (Walton & Hammond 1938), and in human studies (Cawley et al 1954). Additionally, it has been suggested that poor maternal intrauterine growth is associated with reduced weight gain during pregnancy (Hackman et al 1983), and that girls born small for gestational age who remain small have reduced uterine and ovarian size (Ibanez et al 2000). Indeed, in this study maternal size was reduced in the F1 dex mothers at the time of conception.

It has also been proposed that maternal blood pressure might influence fetal growth, indeed in human studies, lower maternal birth weight is associated with an increased risk of hypertension during pregnancy (Klebanoff et al 1999) and higher maternal blood pressure during pregnancy is associated with lower offspring birth weight (Churchill et al 1997, Ferrer et al 2000, Brown et al 2001, Buchbinder et al 2002).

Maternal birth weight is related to offspring blood pressure (Barker et al 2000) and higher maternal blood pressure later in life is also associated with higher offspring blood pressure (Walker et al 1998), suggesting that a programmed increase in blood pressure might have intergenerational effects on fetal growth and subsequent disease. In a recent animal study, blood pressure was higher in the female offspring of rabbits with surgically induced hypertension (Denton et al 2003), suggesting a specific intergenerational effect of higher maternal blood pressure on offspring blood pressure. However, in the study described in this chapter, no differences were found in maternal blood pressure in F1 or F2 female offspring; programmed effects on blood pressure were only seen in males in the F2 generation. Although this suggests that programmed changes in blood pressure are unlikely to play an important role in intergenerational effects in the F2 generation in this model, specific effects acting during pregnancy cannot be excluded.

In view of the known effect of fetal overexposure to glucocorticoids to programme offspring hypercorticosteronaemia (Nyirenda et al 1998), elevated maternal glucocorticoid levels might play a role in the transgenerational programming of birth weight, PEPCK, glucose intolerance and blood pressure. Indeed, non-genetic intergenerational transmission of behavioural and HPA responses to stress, hypothalamic CRH and hippocampal GR expression has been reported in rats (Francis et al 1999). However, in our study, there was no evidence of hypercorticosteronaemia in the F1 dex females, although again this does not rule out a specific effect during pregnancy or the possibility that average levels across a full diurnal rhythm, or peak levels could be increased.

In animal models of diabetes and in models of programming by nutrition, intergenerational effects have been noted on glucose tolerance and the development of the endocrine pancreas (Hoet & Hanson 1999, Martin et al 2000, Fowden & Hill 2001, Van Assche et al 2001). *In utero* exposure to a low protein diet or maternal food restriction results in impaired pancreatic β-cell development in the offspring (Snoeck et al 1990, Dahri et al 1991, Garofano et al 1997, Garofano et al 1998), with impaired glucose tolerance in adulthood (Dahri et al 1991, Garofano et al 1999) and

an inability to increase insulin production during pregnancy (Dahri et al 1995, Blondeau et al 1999). F2 offspring of protein-restricted animals also have abnormal pancreatic development (Hoet & Hanson 1999, Reusens & Remacle 2001). Thus, these studies demonstrate that exposure to an unfavourable uterine environment may affect pancreatic development in a second generation.

Furthermore, second generation effects on glucose tolerance and body weight have been demonstrated in the offspring of females rendered hyperinsulinaemic and obese as a result of rearing on a high carbohydrate milk formula during their suckling period (Laychock et al 1995, Srinivasan et al 2003). These studies suggest that exposure to a hyperinsulinaemic environment *in utero* is sufficient to programme hyperinsulinaemia and obesity in the offspring.

In humans, maternal glucose metabolism can have a profound effect on offspring birth weight, and on later diabetes risk. Maternal blood glucose levels are directly related to fetal weight, including glucose values within the normal range (Farmer et al 1988, Breschi et al 1993) and maternal diabetes is generally associated with fetal overgrowth or 'macrosomia', although the presence of severe complications of the disease can result in intrauterine growth retardation (reviewed in Van Assche et al 2001). Thus, insulin resistance associated with glucose intolerance tends to increase birth weight in the offspring. However, hyperinsulinaemia without glucose intolerance might be a cause of fetal undernutrition (Breschi et al 1993). Indeed, some studies in humans have found that higher maternal fasting insulin is associated with lower offspring birth weight (Breschi et al 1993, Baur et al 1999). In the study described in this chapter, no differences were seen in glucose and insulin levels in the F1 females, arguing against a role for hyperinsulinaemia in the intergenerational effects described.

There were a number of gender differences in this model of programming. F1 dex females did not demonstrate full catch-up growth, unlike the F1 dex males. There were also gender differences in the F2 generation; males appeared to be more susceptible to intergenerational effects than females. F2 dex males had higher blood

pressure and more striking glucose intolerance than the F2 dex females. A number of sex-specific effects have been described in animal models of fetal programming (Hales et al 1996, Dean & Matthews 1999, Kind et al 1999, Lingas et al 1999, Smith & Waddell 2000, Dean et al 2001, Lingas & Matthews 2001, Liu et al 2001, Owen & Matthews 2003). In some paradigms, females have been more sensitive to programming effects; exposure to excess glucocorticoid *in utero* delays puberty in female (but not male) rats (Smith & Waddell 2000) and female offspring of hypertensive female rabbits have elevated blood pressure, whereas males are normotensive (Denton et al 2003). Human studies have revealed some sex differences in the long-term disease risk associated with low birth weight (Forsen et al 2000, Walker et al 2002), or exposure to famine prenatally (Ravelli et al 1999). Such sex-specific effects may represent the differential sensitivity of males and females to programming phenomenon and indeed, stronger programming in females may further amplify a matrilineal pattern of intergenerational inheritance.

In conclusion, this study has demonstrated an intergenerational effect of glucocorticoid programming on birth weight, hepatic PEPCK, glucose tolerance and blood pressure in the second generation in rats. The programming effects appear to be stronger in the F2 generation than in F1 animals, and in male offspring, and are no longer evident in a third generation. However, this experiment has not explored in any detail the potential for parent-specific effects on subsequent generations. Although programmed alterations in maternal physiology might impact upon a developing fetus, leading to intergenerational programmed effects, we have not excluded a paternal effect on offspring phenotype. Although the mother may influence offspring development by genetic or environmental mechanisms, clearly the major paternal influence in this experiment is through genomic mechanisms. The paternal role in intergenerational effects will be addressed in chapter four.

Chapter Four – The influence of paternal phenotype on intergenerational programming effects.

4.1 Introduction

Although there is evidence for a greater maternal influence on offspring characteristics, there is clearly also an influence of the father on offspring birth weight and the later development of cardiovascular disease (Emanuel et al 1992, Davey Smith et al 1997, Klebanoff et al 1998, Magnus et al 2001, Kinra et al 2003, Lawlor et al 2003b) and on type 2 diabetes risk (Mitchell et al 1995, McCarthy et al 1996, Viswanathan et al 1996, Meigs et al 2000, Yajnik et al 2001, Hypponen et al 2003). Indeed, as discussed in chapter one, in the Pima Indian Community, low birth weight is associated with the subsequent development of type 2 diabetes, but only if paternal diabetes is also present.

In chapter three we demonstrated the intergenerational programming effects of in utero overexposure to glucocorticoids on birth weight, PEPCK and glucose tolerance. We have used the same model to investigate whether these transgenerational effects are mediated exclusively via programmed changes in maternal physiology, which would therefore be inherited through the maternal line, or whether there is an effect of paternal phenotype on offspring birth weight and hepatic PEPCK.

4.2 Methods

To investigate the effect of paternal phenotype on the offspring in more detail, a new F1 cohort was generated using the methods described in chapter three. For the F1 breeding, F1 females weighing around 230-250g were timed-mated with F1 males as follows: 8 F1 dex females were timed-mated with F1 dex males (dex/dex crosses) and 8 F1 veh females with F1 veh males (veh/veh crosses), giving 8 litters per group. A further group of 5 F1 dex females were timed-mated with F1 veh males (dex/veh crosses), and 6 F1 veh females with F1 dex males (veh/dex crosses). Females were caged separately during pregnancy and not manipulated in any way; all produced litters. Pups were weighed and culled to eight at birth and weaned at 3 weeks. All offspring were weighed at weaning and monthly thereafter.

At five weeks (F1) or three weeks (F2) of age, male animals from each group (n=8-10) were selected randomly from as many litters as possible. They were culled by cervical dislocation and tissues dissected and snap-frozen on dry ice. Portions of liver were collected into Eppendorfs and placed on wet ice for PEPCK analysis.

Data are expressed as mean ± SEM unless otherwise stated. Data were analysed using unpaired *t* tests, 2-way or repeated measures ANOVA (glucose tolerance tests, longitudinal body weight) where appropriate. Significance was set at P of 0.05 or less.

4.3 Results

4.3.1 Birth weights and postnatal growth

Birth weights were significantly reduced in F1 dex offspring in this cohort (Table 4.1). Females receiving dexamethasone gained less weight during the last week of gestation, but there was no change in gestation length, litter number or the proportion of male and female pups (Table 4.1).

F1 dex males achieved full catch-up growth by 2 months of age and, in contrast to the experiment described in chapter three, F1 females also achieved full catch-up growth by 4 months of age (Figure 4.1).

4.3.2 F1 PEPCK

There were no differences in hepatic PEPCK activity between male F1 dex and F1 veh animals (Table 4.1).

	Dex	Veh	P value
F1 Birth weight (g)	5.34 ± 0.04	6.01 ± 0.07	<0.001
F1 Gestation length (days)	20.6 ± 0.2	21.1 ± 0.2	0.062
F1 Litter number	14.7 ± 0.7	14.8 ± 1.2	0.96
Maternal weight gain (g)	61.8 ± 3.8	88.2 ± 8.4	0.018
PEPCK activity (nmol/min/mg protein)	10.8 ± 0.5	9.9 ± 1.0	0.44

Table 4.1 Data for F1 cohort.

Data are for 10 F1 dex and 9 F1 veh litters. Birth weights for 147 F1 dex and 133 F1 veh pups. PEPCK activity was measured on fresh liver homogenates at 5 weeks on male animals (n=8 F1 dex and 7 F1 veh). Data were analysed by Student's *t* test.

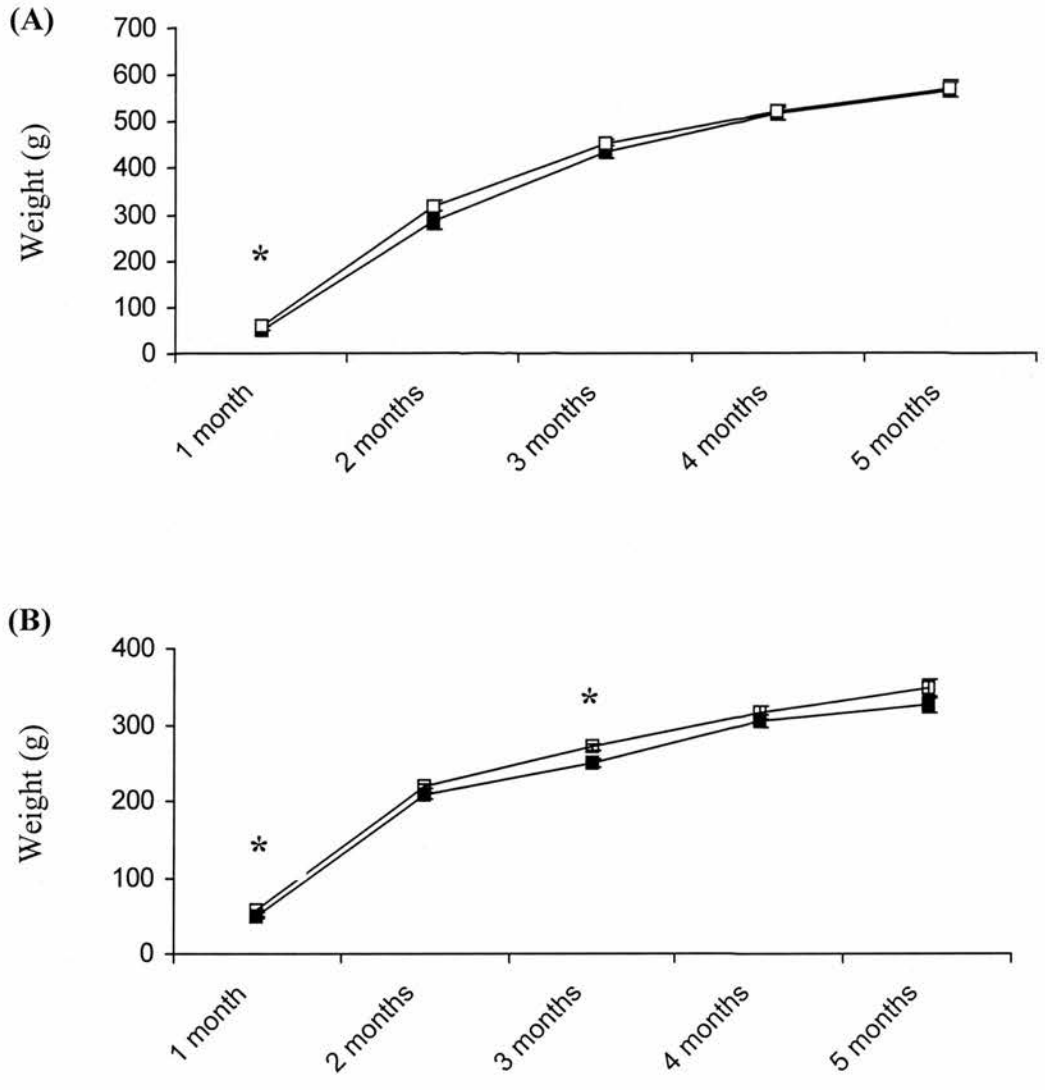


Figure 4.1 Postnatal growth in F1 cohort.

Growth in F1 males (panel A, n= 50 per group at 1 month, 8 per group thereafter) and females (panel B, n=22-25 per group at 1 months, 14-20 at 2-3 months, 6 per group thereafter).

Data were analysed by ANOVA. Post hoc analysis revealed persistent differences in weight between dex and veh males only at weaning, and between dex and veh females only at weaning and 3 months; *denotes $p<0.05$.

□ F1 veh

■ F1 dex

4.3.3 Birth weight and postnatal growth in F2 animals

In contrast to the experiment described in chapter three, in which F1 dex females had been significantly lighter at the time of mating, there were no differences in maternal body weights between the F1 dex and F1 veh females at gestation day E0 in this experiment (Table 4.2). There were no differences in maternal weight gain, gestation length or litter number in any of the breeding groups (Table 4.2).

As in the previous F2 cohort, birth weights were lower in the F2 offspring of dex/dex crosses (F2 dex/dex) when compared with F2 offspring of veh/veh crosses (F2 veh/veh), although the effect was not as marked as before (Table 4.2).

More striking differences were noted in the offspring of crosses between F1 dex and F1 veh males and females. Litter size was more variable than with dex/dex or veh/veh crosses (Table 4.2). Birth weights of the offspring of F1 dex mothers crossed with F1 veh fathers (F2 dex/veh) were not different from those of dex/dex crosses; however birth weights of the offspring of F1 veh mothers crossed with F1 dex fathers (F2 veh/dex) were lower than those in all other groups (Table 4.2).

There were no differences between body weights in any of the groups at weaning, i.e. males and females in all groups had achieved catch-up growth.

4.3.4 PEPCK

As in the previous F2 cohort, hepatic PEPCK activity was elevated in male offspring of dex/dex crosses (F2 dex/dex) when compared with the offspring of veh/veh crosses (Figure 4.2)

PEPCK activity in the F2 offspring of veh mothers and dex fathers (F2 veh/dex) was elevated when compared to the offspring of veh/veh crosses, reaching the same value as that of the dex/dex crosses. PEPCK activity in the offspring of dex mothers and veh fathers (F2 dex/veh) was almost 3 fold higher than that in the offspring of veh/veh crosses (Figure 4.2).

Litter	F2 veh/veh	F2 dex/dex	F2 veh/dex	F2 dex/veh
Mother Father	veh veh	dex dex	veh dex	dex veh
Gestation (days)	22.3 ± 2	22 ± 0	21.5 ± 0.7	22.4 ± 0.2
Litter no.	15.8 ± 0.4	14.8 ± 0.8	13.2 ± 2.2	13.6 ± 2.2
Maternal wt E0	286 ± 7	265 ± 13	282 ± 8	279 ± 10
Maternal wt gain (g)	150 ± 7	146 ± 6	154 ± 13	137 ± 11
Birth wt (g)	6.15 ± 0.05	5.92 ± 0.05	5.71 ± 0.09	5.96 ± 0.08

Table 4.2 F2 data

Data are mean ± SEM. Analysis by ANOVA revealed no differences between the groups for gestation length, litter number, maternal weight at E0 or maternal weight gain. Litters were obtained for 8 veh/veh, 8 dex/dex, 5 dex/veh and 6 veh/dex crosses.

Analysis by ANOVA of birth weights in offspring of veh/veh (n=126), dex/dex (n=116), veh/dex (n=71) and dex/veh (n=62) crosses revealed that birth weights were lower in the offspring of dex/dex crosses (F2 dex/dex) when compared with F2 offspring of veh/veh crosses (F2 veh/veh) ($p<0.05$). Birth weights of the offspring of F1 dex mothers crossed with F1 veh fathers (F2 dex/veh) were also lower than F2 veh/veh ($p<0.05$), but were not different from those of dex/dex crosses. Birth weights of the offspring of F1 veh mothers crossed with F1 dex fathers (F2 veh/dex) were lower than those in all other groups ($p<0.05$).

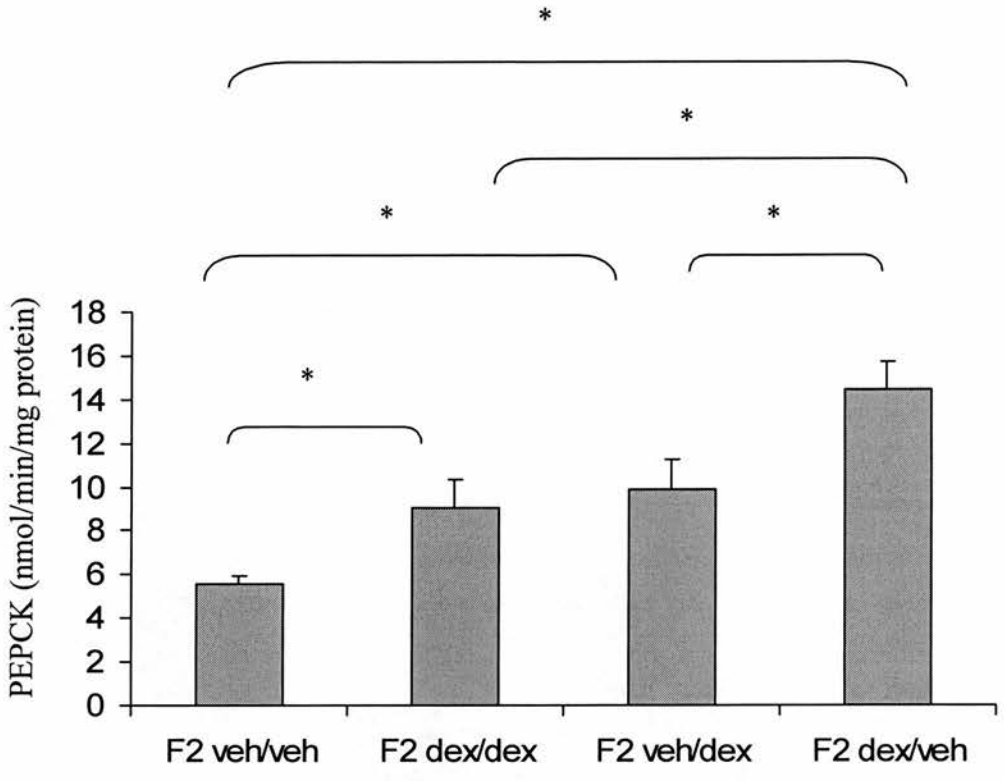


Figure 4.2 Hepatic PEPCK activity in F2 males.

PEPCK activity was measured in fresh liver homogenates at 3 weeks of age. N = 8 per group for F2 dex/dex and F2 veh/veh, 7 per group for F2 veh/dex (veh mother x dex father) and dex/veh (dex mother x veh father). Data were analysed by ANOVA; * $p<0.05$.

4.4 Discussion

The data presented in this chapter confirm an intergenerational effect on birth weight and hepatic PEPCK activity in the dexamethasone programmed rat and additionally, reveal an intriguing paternal effect on birth weight and PEPCK activity in the F2 generation.

Consistent with the findings described in chapter three, birth weight was reduced and hepatic PEPCK activity elevated in F2 dex offspring, compared to F2 veh offspring. F1 and F2 offspring birth weights in this experiment tended to be higher than those in the original, which may reflect the variability of birth weight in the relatively outbred strain of animals used. Indeed, other reports using the same model have reported different birth weights in the F1 offspring (Nyirenda et al 1998, Welberg et al 2001, Cleasby et al 2003a). In addition, F1 females in this cohort achieved full catch-up growth by four months of age, and there were no differences between groups in maternal weight at gestational day E0, excluding a major effect of maternal size in intergenerational programming effects in this model.

In addition to confirming an intergenerational effect of dexamethasone programming, this experiment has demonstrated a significant influence of paternal phenotype on birth weight and PEPCK activity. In the F2 offspring of F1 dex females, there was no additional effect of paternal phenotype on birth weight; birth weights of the offspring of F1 dex females were no different between the groups fathered by F1 dex and F1 veh males. However, in the offspring of dex females, there was a marked effect of paternal phenotype on offspring PEPCK activity, such that the paternal dex phenotype attenuated the increase in hepatic PEPCK activity seen in the offspring of dex mothers and veh fathers. In contrast, in the F2 offspring of F1 veh females, paternal dex significantly reduced birth weight and increased PEPCK.

Most studies of intergenerational effects in animal models of programming have concentrated on the consequences of interventions in the mother (Pinto & Shetty

1995, Francis et al 1999, Martin et al 2000, Patel et al 2001, Denton et al 2003, Srinivasan et al 2003) and second generation effects on glucose tolerance and blood pressure have been demonstrated in the offspring of females crossed with males in the same treatment group (Laychock et al 1995), or untreated males (Denton et al 2003, Srinivasan et al 2003). The authors of these studies have suggested that *in utero* exposure to an adverse maternal environment resulted in programmed alterations in the development of the endocrine pancreas (Laychock et al 1995, Srinivasan et al 2003) and in the renin-angiotensin system (Denton et al 2003).

These effects observed here on birth weight and PEPCK activity in the F2 offspring demonstrate that the father also has an effect on the intergenerational transfer of disease risk in this model. Thus, it would appear that the intergenerational effects described in this study might not be explained entirely by the exposure of the fetus to a programmed adverse maternal environment. Alterations in the DNA sequence are unlikely to occur rapidly enough to explain intergenerational effects that are transmissible from both parents and additionally, the phenotype is attenuated in the third generation. One potential mechanism is 'epigenetic' modification, which may affect the phenotype without changing the DNA sequence, mediated by alterations in DNA methylation and histone modification, which influence transcriptional activity and gene expression (Rakyan et al 2001, Li 2002). In humans, alterations in the expression of imprinted genes can lead to a number of recognised syndromes, including some associated with altered patterns of growth, for example Beckwith-Wiedemann, Prader-Willi and Silver-Russell syndromes (for review see Preece & Moore 2000). Indeed, imprinting of genes has been proposed as the mechanism behind the association of lower offspring birth weight with paternal diabetes in the Pima Indian population (Lindsay et al 2000, Lindsay et al 2002a), and in the parental differences seen in the transmission of class III alleles of the INS-VNTR, which has been associated with type 2 diabetes (Huxtable et al 2000). However, epigenetic modification is not restricted to parentally imprinted genes and recent evidence suggests that epigenetic modifications at some other alleles may not be completely erased during gametogenesis and embryogenesis, potentially resulting in the intergenerational inheritance of the epigenetic state (Roemer et al 1997, Morgan et al

1999). Indeed, one study has demonstrated phenotypic alterations in mice corresponding with differential methylation, which shows transgenerational epigenetic inheritance after both paternal and maternal transmission of the relevant allele, displays parent of origin effects and is influenced by the strain background (Rakyan et al 2003).

How might these epigenetic changes arise? Environmental influences may affect gene expression and have effects on growth (Reik et al 1993, Dean et al 1998, Khosla et al 2001, Young et al 2001, Reik et al 2003). Indeed, maintaining pregnant mice on a diet rich in methyl donors profoundly affects the phenotype in Agouti Yellow mouse pups by increasing methylation of the agouti gene (Waterland & Jirtle 2003). Recent evidence suggests that glucocorticoids may influence gene expression; glucocorticoids regulate DNA demethylation within an enhancer of the rat liver-specific tyrosine aminotransferase (TAT) gene in cell culture, the gene remains demethylated following hormone withdrawal and a stronger response to subsequent glucocorticoid exposure indicates that this demethylation may provide some 'memory' of a regulatory event during development (Thomassin et al 2001). Furthermore, there is some evidence for differential methylation of the GR gene in association with different patterns of maternal behaviour in the rat which have intergenerational consequences (Weaver et al 2002). Thus, glucocorticoids may induce epigenetic modifications, potentially leading to intergenerational effects on fetal growth and later disease risk.

The data also indicate that there is not a straightforward association between birth weight and PEPCK, although offspring with a reduced birth weight have higher PEPCK activity, offspring with the lowest birth weight (F2 veh/dex) were not those with the highest PEPCK activity. Thus, the results suggest that different mechanisms may be operating to reduce fetal size and influence subsequent PEPCK activity. In addition, it is possible that the marked paternal effect on offspring phenotype may be by a different, additional pathway to maternal effects; the maternal influence on offspring may be due to effects on the fetal environment and/ or act via genomic mechanisms.

In conclusion, we have shown an effect of paternal early life experience on offspring characteristics in this animal model of programming. The mechanisms underlying this effect are unclear, but could include inherited epigenetic effects. In support of this hypothesis, there is recent evidence that epigenetic modifications of the genome can be transmitted from both parents, with some studies demonstrating parent of origin effects. Environmental factors, including glucocorticoids, could change the pattern of DNA methylation and chromatin remodelling, perhaps altering the expression of imprinted genes in the placenta, the fetus, or both, affecting fetal growth and later disease risk. Furthermore, these effects could be amplified by the subsequent *in utero* or postnatal environment.

Chapter Five – Diet-induced obesity in the Wistar rat

5.1 Introduction

In animal models and in human studies, obesity appears to be an important amplifier of the programming effect (Phillips et al 1994, Lithell et al 1996, Leon et al 1998, Vickers et al 2000, Vickers et al 2001b, Eriksson et al 2002, Eriksson et al 2003). In human studies and in some animal models, obesity is associated with alterations in glucocorticoid metabolism (Livingstone et al 2000a, Rask et al 2001, Rask et al 2002, Wake et al 2003). Thus, the dexamethasone-programmed rat, in which the programming effect is associated with, and may be dependent on, altered glucocorticoid signalling (Nyirenda et al 1998), may be more susceptible to the development of obesity and its complications. In this chapter we describe the development of a model of diet-induced obesity in Wistar rats, which we have subsequently used to explore the effects of obesity on the dexamethasone-programmed rat (described in chapter six). While developing this model we sought to explore whether dietary-induced obesity induces the same changes in glucocorticoid signalling as those seen in genetic obesity and whether the time course of changes in dietary obesity might provide any insight into the underlying mechanisms.

A number of studies in animal models have sought to determine whether abnormalities in glucocorticoid secretion, action or metabolism may underlie the development of visceral adiposity and the metabolic syndrome. The importance of glucocorticoids in the pathogenesis of obesity is underlined by the observations that the obese phenotype is ameliorated by adrenalectomy or treatment with glucocorticoid receptor antagonists in several animal models (Yukimura et al 1978, Freedman et al 1986, Langley & York 1990, Makimura et al 2000). Extensive studies in the obese Zucker rat have demonstrated increased activity of the HPA axis (White et al 1989, Guillaume-Gentil et al 1990, Havel et al 1996, Timofeeva et al 1999, Livingstone et al 2000a, Livingstone et al 2000b, Mattsson et al 2003), increased reactivation of glucocorticoids by 11β-HSD 1 in adipose tissue, but impaired reactivation in the liver (Livingstone et al 2000a) and increased glucocorticoid

inactivation by hepatic A-ring reductases (Livingstone et al 2000a). Very recently, downregulation of 11β-HSD 1 has been demonstrated in the hippocampus of obese Zucker rats, suggesting a role for alterations in this enzyme in impaired negative feedback on the HPA axis (Mattsson et al 2003). Obesity in leptin deficient *ob/ob* mice is also associated with hypercorticosteronaemia, insulin resistance and decreased hepatic 11β-HSD 1 (Liu et al 2003).

Glucocorticoid action is mediated by low-affinity GR and high-affinity MR; alterations in receptor numbers or binding may affect both peripheral glucocorticoid action and central glucocorticoid feedback. Previous studies of glucocorticoid receptor characteristics in the obese Zucker rat have reported normal or reduced hepatic GR number and affinity (Shargill et al 1987, White & Martin 1990, Langley & York 1992) and reduced MR but normal GR in the hippocampus, which may contribute to the increased HPA activity and appears to be important under conditions of stress (Mattsson et al 2003).

5.2 Methods

Eight female Wistar rats weighing around 250g were timed-mated with Wistar males and caged separately throughout gestation. No manipulations were performed during pregnancy.

At weaning (3 weeks), male offspring were weighed, and four males randomly selected from each litter. Two males from each litter were weaned on to a high fat diet (HF) (Diet D12451, Research Diets, New Brunswick, New Jersey), and two onto a control diet (C) (Diet D01072401, Research Diets) (Section 2.20). Animals were weighed weekly and handled daily for 2 weeks prior to investigation. Eight rats on each diet were investigated after 3 weeks on the diet (Acute) and the remainder after 20 weeks on the diets (Chronic). After investigation at each time point, the animals were culled by decapitation between 0900 h and 1100 h following an overnight fast, and trunk blood was collected. Tissues were dissected immediately, weighed and snap-frozen on dry ice.

In a separate experiment, 12 male Wistar rats were commenced on the high fat and 12 on the control diet at 6 weeks of age. After 24 hours on the diets, 6 control and 6 high fat fed animals were weighed, sacrificed and tissues dissected as before; the remaining 6 animals in each group were investigated after 72 hours on the diets.

Rats in the acute group underwent glucose tolerance testing at 3 weeks and those in the chronic group at 20 weeks. Glucose was determined by the enzymatic (hexokinase) method. Plasma insulin and leptin concentrations were determined by ELISA. Triglycerides and non-esterified fatty acids were determined on plasma from trunk blood samples using kits by Dr Philip Wenham, Department of Biochemistry, Western General Hospital. Plasma corticosterone was measured by RIA on tail nick blood samples taken at 0800 h and 1900 h.

11β-HSD 1 activity was estimated by conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone and analysed by TLC (liver, omental and subcutaneous fat from acute and chronic experiments) or HPLC (liver and fat depots from 24/72 hour experiments). 5β-reductase was measured by conversion of [^{3}H]-corticosterone to [^{3}H]-5β-tetrahydrocorticosterone and analysed by HPLC.

mRNA was quantified by Northern blot (hepatic 5α- and 5β-reductase) or real-time PCR (11β-HSD 1 and GR in all depots; 5α-reductase in omental fat).

Data are expressed as mean ± SEM unless otherwise stated. Data were analysed using unpaired *t* tests, 2-way or repeated measures ANOVA (glucose tolerance tests, longitudinal body weight) where appropriate. Significance was set at P of 0.05 or less. N=8 for control and high fat at 3 weeks, 7 control and 8 high fat at 20 weeks, and 6 for both groups at 24 and 72 hours.

5.3 Results

5.3.1 Body weight

After 24 hours, 72 hours and 3 weeks, HF rats showed no differences in body weight or in organ weight (Table 5.1), however from 60 days HF rats had higher body weights (Figure 5.1). At culling, HF rats had increased retroperitoneal fat weight (Table 5.1), but no differences in the weight of other organs measured.

5.3.2 Glucose tolerance and lipids

After 3 weeks, HF rats were glucose intolerant, but had no difference in plasma insulin levels (Figure 5.2). After 20 weeks, HF rats remained hyperglycaemic and in addition, were markedly hyperinsulinaemic (Figure 5.2).

HF rats showed a trend towards lower plasma triglycerides (TG) after 3 weeks and a significant decrease after 20 weeks. There were no differences in non-esterified fatty acids (FFA) at either time point (Table 5.1).

There were no significant differences in plasma leptin at 3 or 20 weeks, although there was a trend towards an increase in HF animals by 20 weeks (Table 5.1).

5.3.3 HPA axis

There were no differences in basal or peak plasma corticosterone levels after 3 weeks (Figure 5.3). At 20 weeks, there were no differences in basal corticosterone levels, but HF rats had lower peak plasma corticosterone (Figure 5.3).

There were no differences in mRNA for hepatic or omental fat GR at 3 or 20 weeks (Table 5.2).

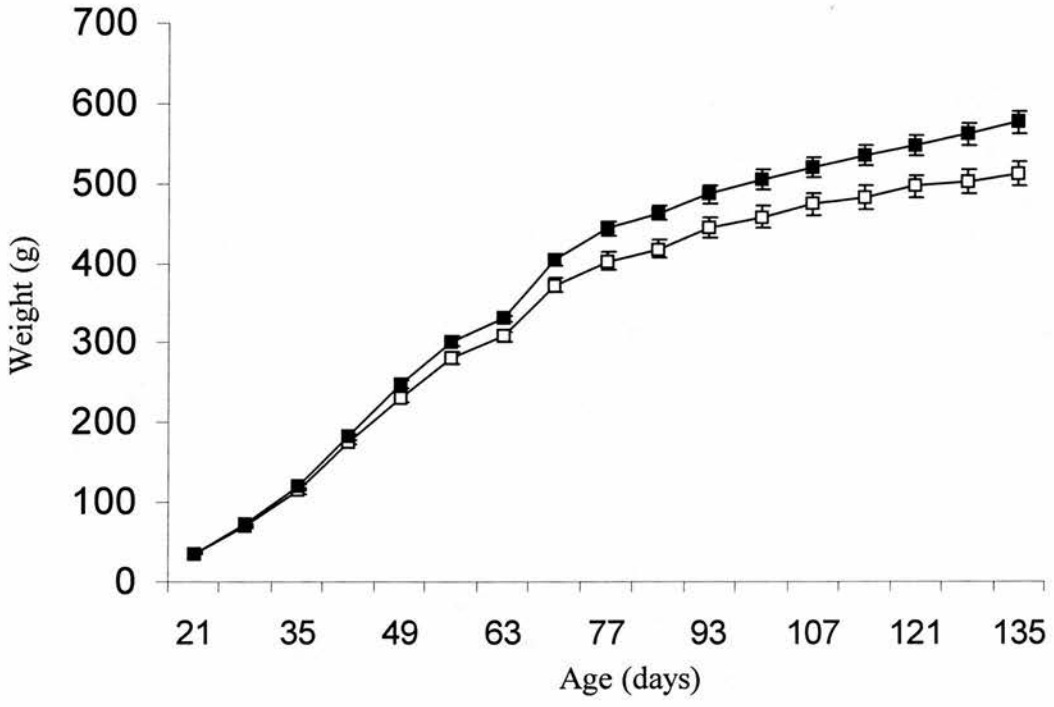


Figure 5.1 Body weights of rats on high fat (HF) and control (C) diets. Data were analysed by repeated measures ANOVA. Rats on HF diet were significantly heavier from 60 days ($p = 0.03$). N=7 control and 8 high fat.

□ Control diet

■ High Fat diet

	3 weeks		20 weeks	
	Control	High fat	Control	High fat
BW (g)	188 ± 10	194 ± 9	541 ± 20	618 ± 17 †
RP fat wt (g)	1.1 ± 0.2	1.5 ± 0.4	12.9 ± 0.9	21.7 ± 1.9†††
Leptin ng/ml	0.7 ± 0.1	0.8 ± 0.1	2.4 ± 0.4	3.2 ± 0.2
Serum (mM)				
Fatty acids	0.49 ± 0.06	0.43 ± 0.02	0.50 ± 0.03	0.49 ± 0.04
Triglycerides	1.3 ± 0.2	0.9 ± 0.1	2.0 ± 0.4	1.3 ± 0.1 †

Table 5.1 Weight and plasma parameters for animals on HF and C diets.
Body weight (BW), retroperitoneal fat weight (RP) and plasma parameters from animals at 3 weeks (n= 8 C and 8 HF) and 20 weeks (n= 7 C and 8 HF).
Data are the mean ± SEM. † indicates a significant difference between high fat and control groups at 20 weeks (†, $P<0.05$; †††, $P<0.005$).

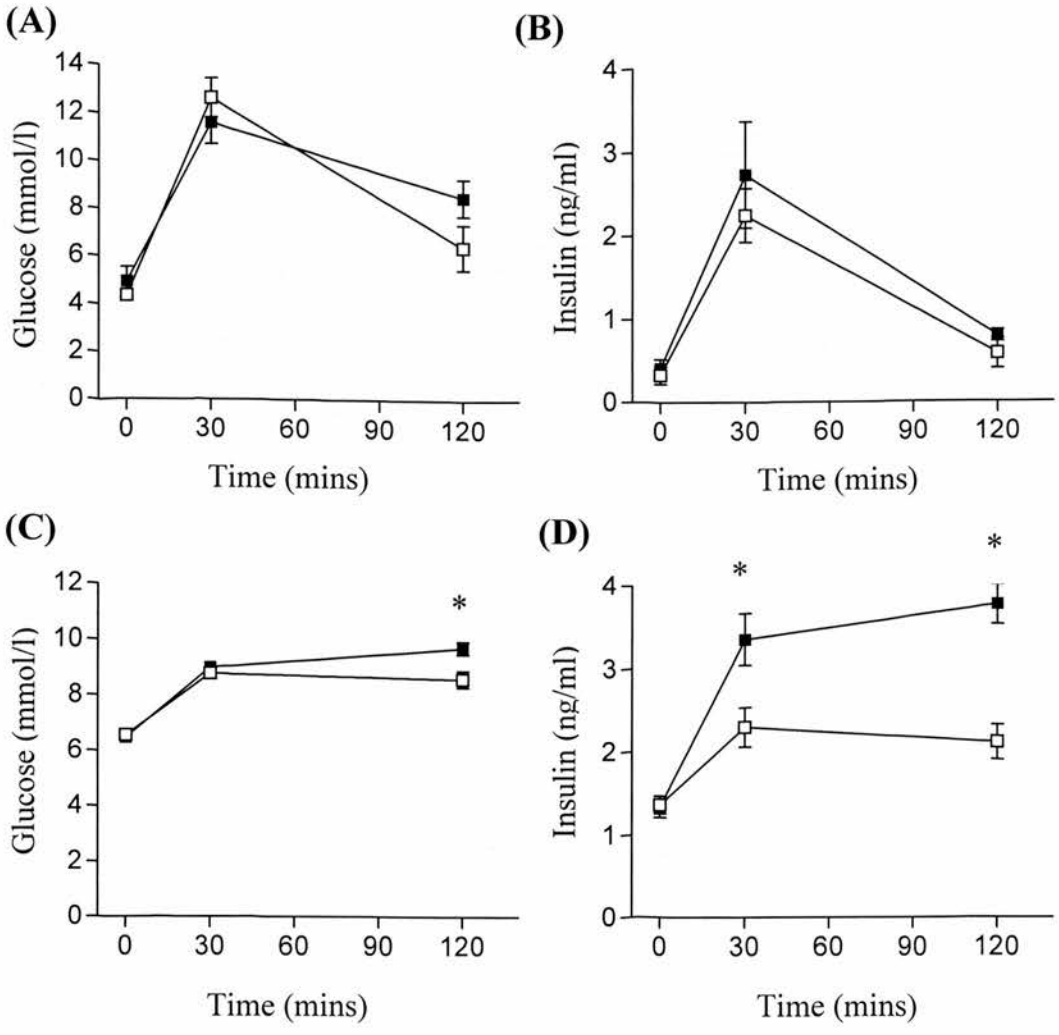


Figure 5.2 Glucose tolerance test after 3 and 20 weeks on HF and C diet. Plasma glucose (panel A) and insulin (panel B) after 3 weeks (n=8 per group), plasma glucose (panel C) and insulin (panel D) after 20 weeks (n= 7 C and 8 HF).

Data were analysed by ANOVA * denotes $p<0.05$ comparing HF and C.

□ Control diet

■ High Fat diet

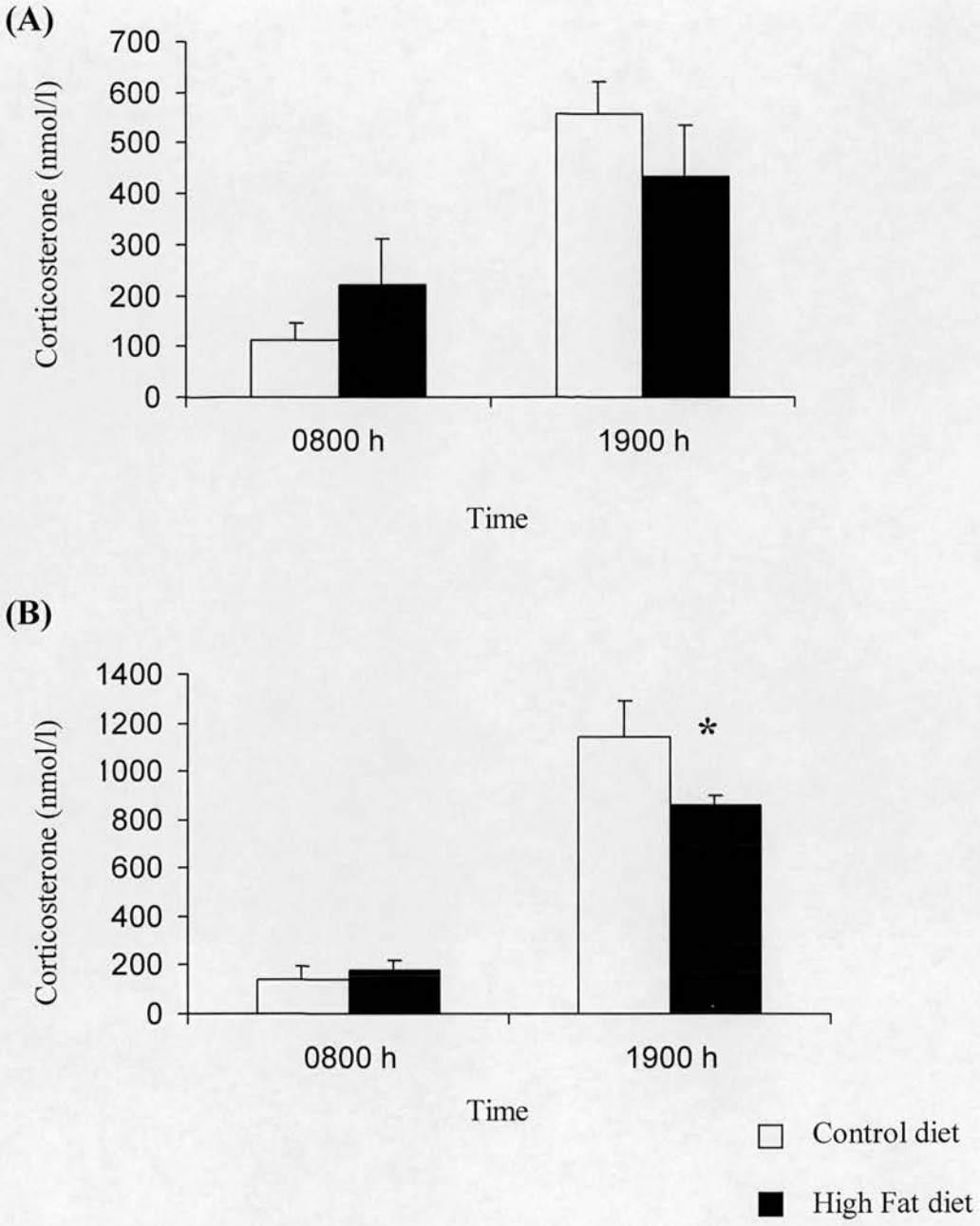


Figure 5.3 Plasma corticosterone levels.

Plasma levels at 0800 h (basal) and 1900 h (peak) after 3 weeks (n= 8 per group) (panel A) and 20 weeks (n= 7C and 8 HF) (panel B). Analysis by ANOVA showed no effect of diet or time on basal corticosterone levels, but an effect of HF to decrease peak levels by 20 weeks ($p<0.05$). Peak corticosterone levels at 20 weeks were higher than at 3 weeks in both HF and C ($p<0.05$) (*denotes $p<0.05$ comparing HF and C).

5.3.4 11β-HSD 1

At 24 and 72 hours there were no differences in hepatic or omental fat 11β-HSD 1 activity between HF and C animals, although after 72 hours there was a trend towards a decrease in activity in subcutaneous fat from HF rats (Figure 5.4).

By 3 weeks, HF rats had lower 11β-HSD 1 activity in liver, subcutaneous and omental fat (Figure 5.5). After 20 weeks, differences in 11β-HSD 1 activities were no longer apparent (Figure 5.5).

There were no differences at 3 or 20 weeks in mRNA levels for hepatic and omental fat 11β-HSD 1 (Table 5.2)

5.3.5 A-ring reductases[1]

HF rats had higher hepatic 5β-reductase activity after 24 hours on the diet, and a trend towards a decrease at 72 hours which did not reach significance. The increase in activity noted at 24 hours was maintained at 3 weeks (Figure 5.6).

After 20 weeks, there was no difference in hepatic 5β-reductase activity (Figure 5.6).

There were no differences at 3 or 20 weeks in mRNA levels for hepatic and omental fat 5α-reductase or hepatic 5β-reductase (Table 5.2).

[1] Measurement of 5α-reductase mRNA in omental fat by real time PCR was carried out by Caroline Elferink, and measurement of hepatic 5β-reductase activity in tissues from 3 and 20 weeks was performed by Lisa Reidy, both visiting undergraduates in the Endocrine Unit.

	3 weeks		20 weeks	
	Control	High fat	Control	High fat
GR mRNA				
Liver	1.04 ± 0.03	0.99 ± 0.02	0.98 ± 0.01	1.01 ± 0.03
Omental fat	1.02 ± 0.08	1.00 ± 0.06	0.98 ± 0.05	0.95 ± 0.10
11β-HSD 1 mRNA				
Liver	0.92 ± 0.09	0.95 ± 0.04	1.06 ± 0.04	0.97 ± 0.02
Omental fat	0.93 ± 0.04	1.05 ± 0.07	0.93 ± 0.14	0.99 ± 0.06
5α-reductase mRNA				
Liver	1.77 ± 0.06	1.95 ± 0.10	1.67 ± 0.12	1.77 ± 0.10
Omental fat	1.07 ± 0.04	1.16 ± 0.11	0.86 ± 0.07	0.93 ± 0.04
5β- reductase mRNA				
Liver	1.69 ± 0.09	1.76 ± 0.08	2.62 ± 0.12	2.24 ± 0.17

Table 5.2 11β-HSD 1, GR and A-ring reductase mRNA data.

GR and 11β-HSD 1 mRNAs were quantified by real time PCR and are expressed normalised to cyclophilin.

5α-reductase and 5β-reductase mRNAs were quantified by Northern Blot in liver and are expressed normalised to U1. 5α-reductase mRNA was quantified by real time PCR in omental fat, and is expressed normalised to cyclophilin.

N= 8 per group at 3 weeks and 7 C and 8 HF at 20 weeks. Data are mean ± SEM and were analysed by Student's *t* test.

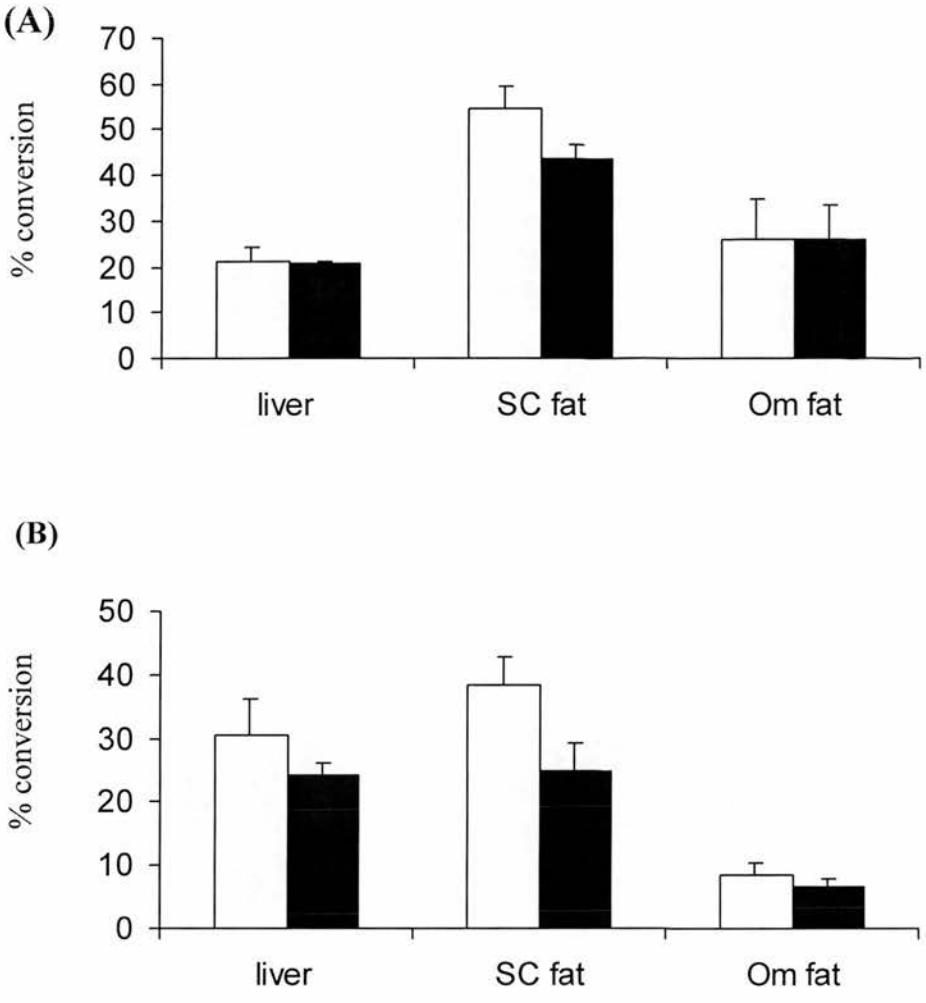


Figure 5.4 Tissue 11β-HSD 1 activity after 24 and 72 hours on HF and C diets.

11β-HSD 1 activity was measured in tissue homogenates, expressed as percent conversion of corticosterone to 11-dehydrocorticosterone after 24 hours (panel A) and 72 hours (panel B). N= 6 per group.

SC = subcutaneous fat; Om = omental fat

Analysis by ANOVA revealed a trend towards a decrease in 11β-HSD 1 activity in subcutaneous fat after 72 hours (p=0.058).

□ Control diet

■ High Fat diet

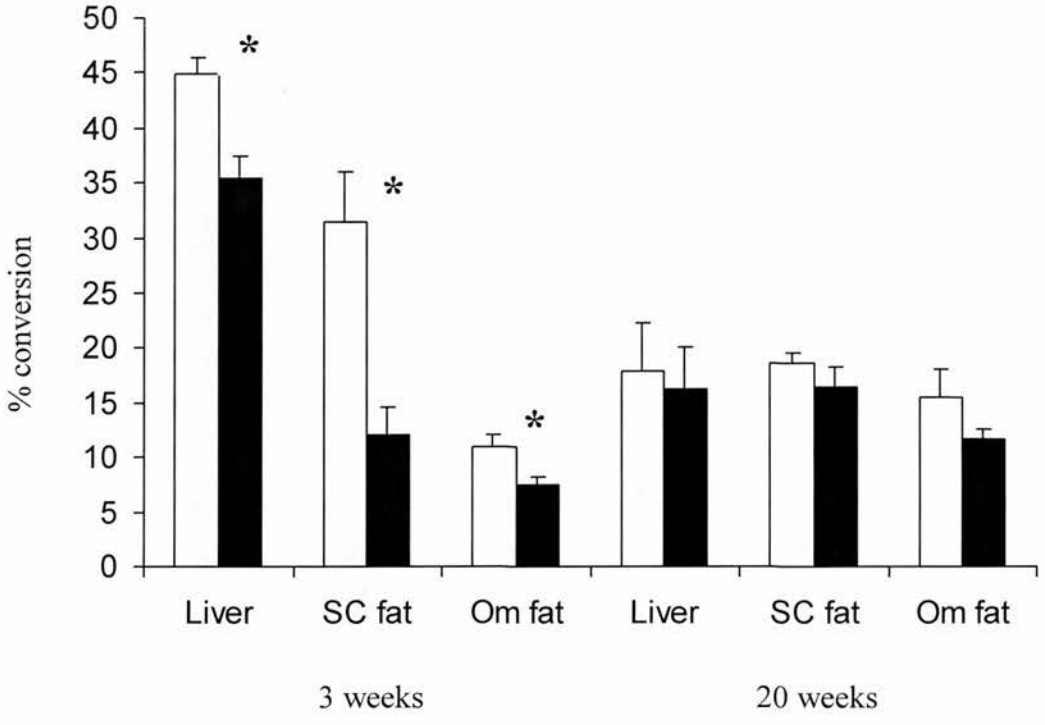
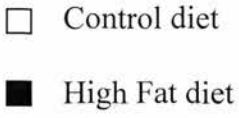


Figure 5.5 Tissue 11β-HSD 1 activity after 3 and 20 weeks on HF and C diets. 11β-HSD 1 activity was measured in tissue homogenates, expressed as percent conversion of corticosterone to 11-dehydrocorticosterone. N= 8 per group at 3 weeks and 7 C and 8 HF at 20 weeks.

SC = subcutaneous fat; Om = omental fat.

Data were analysed by Student's *t* test *denotes $p<0.05$ comparing HF and C.



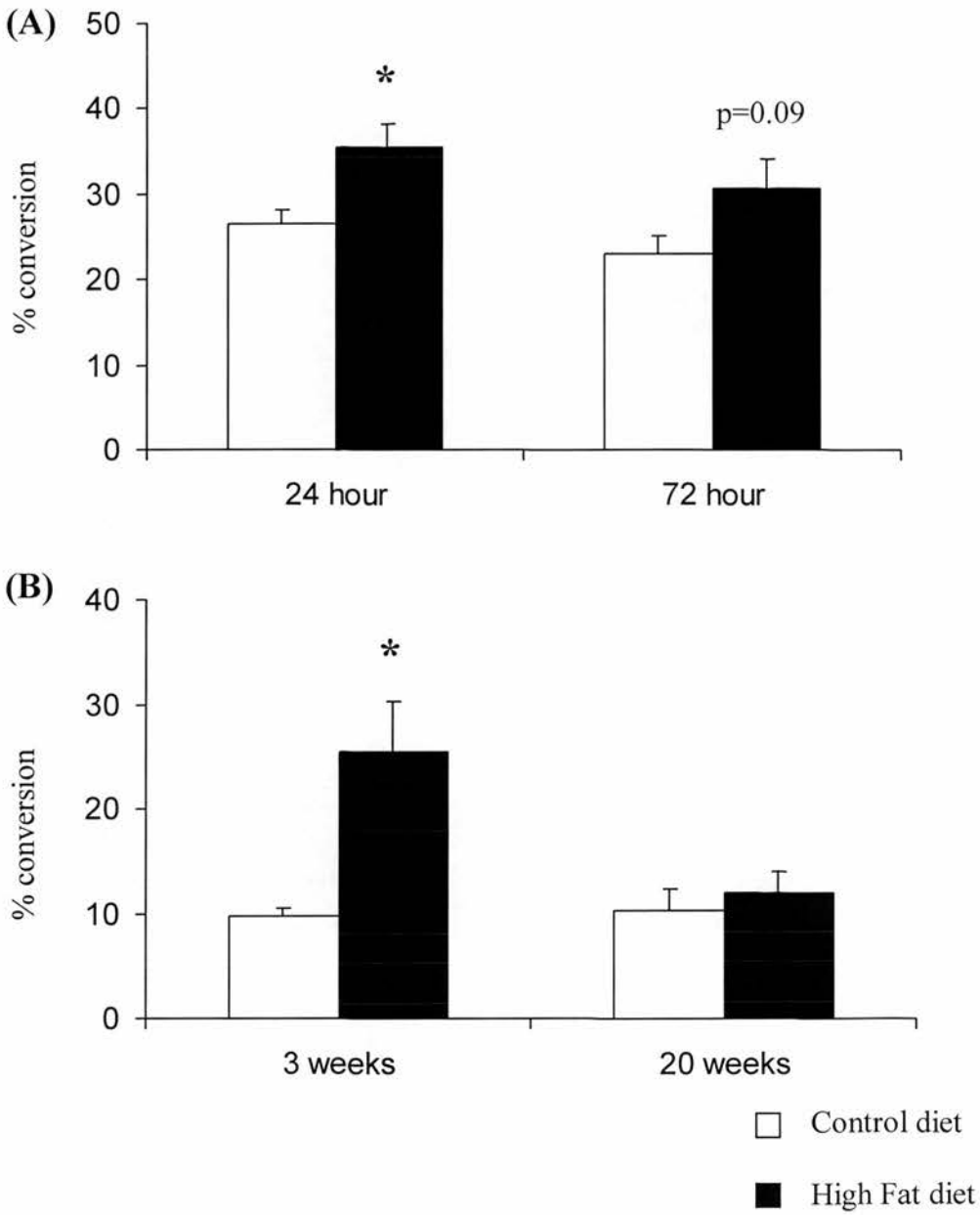


Figure 5.6 Hepatic 5β-reductase activity.

5β-reductase activity after 24 and 72 hours (panel A) and after 3 and 20 weeks (panel B). 5β-reductase was measured in tissue homogenates, expressed as percent conversion of corticosterone to 5β-tetrahydrocorticosterone.

Analysis by ANOVA showed an effect of HF diet to increase 5β-reductase activity at 24 hours (n=6 per group) and 3 weeks (n=8 per group), (* denotes $p<0.05$ comparing HF and C), and a trend to increase activity at 72 hours ($p=0.09$, n=7 C and 8 HF).

5.4 Discussion

These results demonstrate that short-term high fat feeding in rodents is associated with the development of insulin resistance and acute changes in glucocorticoid metabolising enzymes, in the absence of obesity. In contrast, rodents maintained on a long-term high fat diet are obese and insulin resistant, with altered activity of the HPA axis, but no longer exhibit such striking changes in glucocorticoid metabolising enzymes. Thus, the model does not show the same alterations in glucocorticoid metabolism that are observed in genetic models of obesity such as the obese Zucker rat (Livingstone et al 2000a), but does provide some insights into the regulation of these enzymes. In addition, there were no changes in GR mRNA in liver or omental fat at either time point, in contrast to the alterations in hepatic GR reported in the insulin resistant dexamethasone-programmed rat (Nyirenda et al 1998) and in the obese Zucker rat (White & Martin 1990).

In this study, long-term high fat feeding was associated with the development of obesity and profound insulin resistance without basal hyperglycaemia, in agreement with results from some (Tannenbaum et al 1997, Chalkley et al 2002, Commerford et al 2002), but not all (Wang et al 1998), previous studies. The mechanisms by which chronic high fat feeding induces insulin resistance remain unclear, but may include the failure of insulin to suppress elevated muscle and liver lipid availability, with a consequent reduction in insulin mediated glucose disposal in muscle and reduced suppression of hepatic gluconeogenesis (Oakes et al 1997). Additionally, studies have shown that high fat feeding is associated with reduced insulin-mediated suppression of glucose-6-phosphatase and a chronic reduction in hepatic glucokinase (Oakes et al 1997).

HF rats had a marked increase in the amount of retroperitoneal fat after 20 weeks. Human studies have demonstrated that the preferential accumulation of visceral fat is a more important predictor of increased morbidity and mortality than body mass index or total body adipose mass (Montague & O'Rahilly 2000). Indeed, selective reduction in visceral adiposity in humans by diet and exercise is associated with an

improved cardiovascular risk profile (Riches et al 1999) and in animal models, surgical removal of visceral fat (epididymal and perinephric fat pads) reverses hepatic insulin resistance and hepatic glucose output (Barzilai et al 1999).

The metabolic syndrome in humans is associated with hypertriglyceridaemia, however in this model, HF rats showed a reduction in plasma TG levels after 20 weeks, and no change in plasma FFAs. Although some studies have shown increased serum FFA in rats on a high fat diet (Tannenbaum et al 1997), others have shown decreased FFA and TG on high fat feeding (Kraegen et al 1991, Morin et al 1997, Vickers et al 2001a, Chalkley et al 2002, Hegarty et al 2002, Picard et al 2002, Chen & Nyomba 2003); this has been attributed to enhanced clearance of fatty acids into muscle (Hegarty et al 2002). In addition, it has been suggested that fasting FFA levels are extremely variable and appear to correlate poorly with measures of insulin resistance (Steinberg et al 1997).

There were no differences in basal plasma corticosterone levels at 3 or 20 weeks. In rodents, maintenance on a high fat diet led to increased basal plasma corticosterone levels at 7 days in one study (Tannenbaum et al 1997), although another study did not find this (Kamara et al 1998). Both studies failed to show any difference in basal corticosterone levels after 21 days (Tannenbaum et al 1997, Kamara et al 1998). In our study, peak plasma corticosterone levels were significantly reduced after 20 weeks on the high fat diet. Although short exposure (3 days) to a high fat diet does not alter peak corticosterone levels (Kamara et al 1998), peak levels have been shown to decline after 5 weeks in rats maintained on a diet containing more than 30% fat (Wang et al 1998). Additionally, in rats, stress induced HPA axis activity is altered by short- and long-term exposure to a high fat diet (Tannenbaum et al 1997, Kamara et al 1998), suggesting that high fat diets may act as a model of chronic stress (Tannenbaum et al 1997). Thus in rodents, high fat diets may influence HPA activity, without increasing circulating glucocorticoid levels. Likewise, in human studies, obesity, increased visceral fat mass and derangement of metabolic variables such as insulin, glucose, and triglycerides, are associated with abnormal HPA axis variability, with a low diurnal cortisol variation, and normal or low peak cortisol

levels (Rosmond et al 1998, Bjorntorp et al 1999, Rask et al 2001), although there is evidence for HPA axis activation (Marin et al 1992, Pasquali et al 2002).

The metabolism of glucocorticoids by the isoenzymes of 11β-HSD is important in modulating the access of glucocorticoids to the glucocorticoid receptors GR and MR, thereby determining local glucocorticoid effects (Seckl & Walker 2001). Reactivation of corticosterone or cortisol from their inactive metabolites by 11β-HSD 1 is predicted to increase tissue exposure to active glucocorticoid. Previous studies in rodent models and in humans have demonstrated that obesity is associated with altered 11β-HSD 1 activity, which is increased in adipose tissue (Livingstone et al 2000a, Rask et al 2001, Paulmyer-Lacroix et al 2002, Rask et al 2002), but decreased in liver (Livingstone et al 2000a, Rask et al 2001, Rask et al 2002). Furthermore, there is evidence that altered 11β-HSD 1 activity may play a key role in the pathogenesis of visceral obesity and the metabolic syndrome. Transgenic mice over-expressing 11β-HSD 1 specifically in adipose tissue develop visceral obesity and features of the metabolic syndrome, without an increase in circulating corticosterone levels (Masuzaki et al 2001). In contrast, mice homozygous for a targeted disruption of the 11β-HSD 1 gene exhibit attenuated gluconeogenic responses to stress, resist hyperglycaemia and the development of visceral obesity on exposure to a high fat diet and have improved hepatic insulin sensitivity and glucose tolerance (Kotelevtsev et al 1997, Morton et al 2001).

In this study, exposure to a high fat diet for 3 weeks was accompanied by a down-regulation of 11β-HSD 1 activity in liver and adipose tissue. Although no difference in 11β-HSD 1 activity was evident at 24 hours, there was a clear trend for a down-regulation in activity in subcutaneous fat at 72 hours. In addition, there was an acute increase in the activity of hepatic 5β-reductase in HF animals at 24 hours, which was maintained at 3 weeks. This combination of decreased hepatic 11β-HSD 1 and increased hepatic 5β-reductase activity is predicted to reduce local glucocorticoid concentrations and is in agreement with findings in humans and in obese Zucker rats (Livingstone et al 2000a, Rask et al 2001, Rask et al 2002). The resultant increase in the glucocorticoid metabolic clearance rate (MCR) may however, result in increased forward drive to the HPA axis to compensate for this, and to HPA axis activation.

Thus, short-term exposure to a high fat diet results in changes in glucocorticoid metabolism, before the development of obesity, which may act acutely to protect the animal from the metabolic consequences of a high fat diet.

Although in the Zucker rat, obesity is associated with permanent down regulation of hepatic 11β-HSD 1, in this model, we observed no difference in hepatic 11β-HSD 1 activity or mRNA levels after 20 weeks and 5β-reductase activity was normalised. Additionally, in the longer term, the suppression of 11β-HSD 1 activity was no longer evident in adipose tissue. This failure to maintain suppression of adipose 11β-HSD 1 following long-term exposure to a high fat diet may be important in the pathogenesis of the metabolic complications of high fat feeding. Indeed, it is possible that susceptibility to visceral obesity and its metabolic consequences may in part, be determined by the ability to down-regulate adipose 11β-HSD 1.

In transgenic mice, overexpression of 11β-HSD 1 in visceral fat resulted not only in increased adipose tissue concentrations of corticosterone, but also in increased levels of corticosterone and free fatty acids in the hepatic portal vein (Masuzaki et al 2001). Such an increase in the delivery of glucocorticoids and free fatty acids to the liver may further contribute to hepatic insulin resistance. In our model, normalisation of adipose 11β-HSD 1 activity, together with increased fat mass may have similar consequences. This effect is likely to be further compounded by the normalisation of hepatic 11β-HSD 1 and 5β-reductase activities, with a consequent increase in local hepatic glucocorticoid concentration.

The mechanisms by which these enzymes are regulated remain unclear, but candidates include resistance to insulin-mediated regulation, and secondary effects of adipose products. Factors influencing 11β-HSD 1 include glucocorticoids, TNFα, thyroid hormones, sex steroids, GH, IGF-1, insulin and cytokines (Seckl & Walker 2001). Chronic stress or elevated glucocorticoid levels are associated with reduced 11β-HSD 1 activity (Jamieson et al 1999a). In addition, both PPARα and PPARγ agonists attenuate 11β-HSD 1 activity (Hermanowski-Vosatka et al 2000, Berger et al 2001). Treatment of *ob/ob* mice (which have reduced 11β-HSD 1 activity in liver),

with leptin increased hepatic 11β-HSD 1 mRNA and activity, in addition to reducing body weight, plasma glucose and corticosterone (Liu et al 2003). Furthermore, leptin increased 11β-HSD 1 mRNA and activity in isolated hepatocytes, an effect which appeared to be mediated via the leptin receptor (Liu et al 2003).

Although we found alterations in 11β-HSD 1 activity in liver, omental and subcutaneous fat after 3 weeks of high fat feeding, there were no differences in 11β-HSD 1 mRNA in liver and omental fat. Studies in human adipocytes and in a hepatoma cell line have shown down-regulatory effects of CRH and ACTH (Friedberg et al 2003) and IGF-1 (Voice et al 1996) on 11β-HSD 1 activity, without any change in mRNA levels, suggesting that some effects on 11β-HSD 1 are exerted post-transcriptionally. The reason for the discrepancy between enzyme activity and mRNA levels of hepatic 5β-reductase is also unknown. The 5β-reductase enzyme which has been purified from human liver shares approximately 75% homology with that of the rat (Charbonneau & The 2001) and catalyses the transformation of 4-androstenedione, testosterone, progesterone and 17α-hydroxyprogesterone in addition to glucocorticoids and mineralocorticoids, which appear to be poorer substrates (Charbonneau & The 2001). Despite the apparent poor affinity of this enzyme for glucocorticoids, there is a higher abundance of 5β-reduced than 5α-reduced glucocorticoid metabolites and it is possible that there is an as yet undiscovered second 5β-reductase gene. Alternatively, the RNA may be rapidly degraded; indeed the enzyme possesses a sequence motif (ATTTA) within the 3' untranslated region, which is known to be associated with instability and rapid degradation of RNA (Shaw & Kamen 1986).

In this study we have demonstrated that changes in 11β-HSD 1 and 5β-reductase activities are an early change accompanying high fat feeding in rodents, and are seen before the development of obesity. The changes in 11β-HSD 1 appear to be independent of insulin resistance, as they are not maintained in the long term, despite a marked decline in insulin sensitivity. Indeed, these findings support a number of other studies suggesting that changes in 11β-HSD 1 are not due to hyperinsulinaemia

or insulin resistance alone; studies using metformin and PPARγ agonists have shown that insulin sensitisation alone is insufficient to normalise the changes in 11β-HSD 1 enzyme activity in the obese Zucker rat (Livingstone et al 2000b), although high dose PPARγ agonists can attenuate adipose tissue 11β-HSD 1 activity in *db/db* mice (Berger et al 2001). Furthermore, in another rodent model, in which antenatal dexamethasone treatment is associated with the development of insulin resistance in adulthood, no changes are seen in hepatic 11β-HSD 1 mRNA (Nyirenda et al 1998) and in a recent case-control study of lean men with hyperglycaemia and insulin resistance, no changes were found in adipose tissue 11β-HSD 1 (Andrews et al 2002).

In the obese Zucker rat, adrenalectomy reversed the tissue-specific alterations in 11β-HSD 1 (Livingstone et al 2000b), however adrenalectomy also normalises many of the other features of the phenotype including obesity, insulin resistance and hyperinsulinaemia. In lean Zucker rats, adrenalectomy had no effect on 11β-HSD 1 activity in liver, but increased activity in adipose tissue (Livingstone et al 2000b); it is therefore unlikely that the hypercorticosteronaemia alone is responsible for the alterations in 11β-HSD 1 observed in the obese Zucker rat. In our study, acute changes in glucocorticoid metabolising enzymes were noted in the absence of increased plasma corticosterone levels, suggesting that circulating glucocorticoid levels may not play a major role in the regulation of these enzymes in animals on a high fat diet.

Hepatic and omental fat GR mRNA levels were unchanged with high fat feeding in both the short- and long-term experiments. One previous study has reported decreased hypothalamic GR after one week on a high fat diet, although this difference was not maintained after 9 or 12 weeks (Tannenbaum et al 1997). No changes were noted in GR numbers in the hippocampus, frontal cortex or pituitary (Tannenbaum et al 1997). In the obese Zucker rat, previous studies have reported normal or reduced hepatic GR number and affinity (Shargill et al 1987, White & Martin 1990, Langley & York 1992) and reduced MR but normal GR in the hippocampus (Mattsson et al 2003). In contrast, in the dexamethasone-programmed

rat, another rodent model associated with HPA axis activation (Levitt et al 1996) and insulin resistance (Nyirenda et al 1998), although without the development of obesity, GR is again altered in a tissue-specific manner; but in this model is permanently upregulated in liver and visceral fat (Nyirenda et al 1998, Cleasby et al 2003a, Cleasby et al 2003b), and reduced in the hippocampus and certain muscle types (Levitt et al 1996, Welberg et al 2001, Cleasby et al 2003a). In transgenic mice overexpressing 11β-HSD 1 in visceral fat, no alterations were found in GR mRNA in mesenteric or subcutaneous fat (Masuzaki et al 2001).

The lack of any effect on GR mRNA expression of high fat feeding in Wistar rats and in the adipose 11β-HSD 1 overexpressing mice, together with the different and tissue-specific alterations observed in the obese Zucker and dexamethasone-programmed rats, suggests that the changes in GR are unlikely to be mediated by insulin resistance. Further evidence for this comes from recent studies in the dexamethasone-programmed rat, showing that manipulation of circulating corticosterone and insulin sensitisation are insufficient to normalise the programmed changes in peripheral GR, suggesting that they are not secondary to the metabolic derangement, but may be a primary mechanism underlying the metabolic dysregulation in this model (Cleasby et al 2003b). However, it is possible that the long term changes in HPA axis activity noted in this model of high fat feeding are mediated by changes in GR or MR in central feedback sites, which we have not explored in this study.

In conclusion, high fat feeding in Wistar rats leads to obesity and insulin resistance. Short-term exposure to a high fat diet induces alterations in glucocorticoid metabolism, with decreased 11β-HSD 1 activity in liver and fat depots, and increased hepatic 5β-reductase activity, in the absence of hyperinsulinaemia, hypercorticosteronaemia or changes in GR. These alterations are not however, maintained in the longer term, despite the development of obesity and insulin resistance. This study therefore demonstrates that high fat feeding in Wistar rats is not associated with the same changes in glucocorticoid metabolism as are seen in genetic models of obesity, but does providing further support for previous studies which have suggested that the alterations in glucocorticoid metabolism in models of

obesity are not mediated by insulin resistance, hyperinsulinaemia or hypercorticosteronaemia (Livingstone et al 2000b).

Chapter Six – The effects of obesity in the dexamethasone programmed rat

6.1 Introduction

The development of obesity appears to be an important factor modifying the risk of later disease associated with low birth weight. Those individuals with the lowest birth weight but highest current weight have the highest total body fat (Gale et al 2001) and are at increased risk of developing insulin resistance, type 2 diabetes and high blood pressure (Phillips et al 1994, Lithell et al 1996, Leon et al 1998, Eriksson et al 2002, Eriksson et al 2003). In human studies, prenatal famine exposure is linked to decreased glucose tolerance in adulthood, particularly in those who become obese (Ravelli et al 1998) and in the Leningrad siege study, a positive association between obesity and blood pressure was found, with a relationship that was strongest in those exposed to malnutrition *in utero* (Stanner et al 1997, Stanner & Yudkin 2001).

The importance of obesity as an amplifier of programming effects has been explored in animal models in which antenatal nutritional manipulations have resulted in offspring of low birth weight and postnatal growth failure. Rats exposed to maternal undernutrition throughout gestation and maintained on a hypercaloric diet from weaning developed hyperphagia, profound obesity, hyperglycaemia, hyperinsulinaemia, hyperleptinaemia and hypertension (Vickers et al 2000, Vickers et al 2001b).

We have explored the effects of obesity on the phenotype of the dexamethasone-programmed rat, using the model of high fat feeding developed in chapter five.

6.2 Methods

Male F1 pups bred in the programming experiment described in chapter four were used. High fat and control diets were used as described in chapter five (Section 2.20). At three weeks of age, 19 F1 dex pups were weaned onto the high fat diet (DHF), and 15 onto the control diet (DC). Fourteen F1 veh animals were weaned onto high fat (VHF) and 14 onto control (VC) diets. Animals were used from all F1 litters (n=8 litters per group), and in all cases experimental cohorts included males selected randomly from as many litters as possible. Animals were caged in groups of four or five, with *ad libitum* access to the respective diets. All were weighed at weaning and 2-weekly thereafter.

Glucose tolerance tests were conducted at 15 weeks and 6 months on 8-10 animals from each group. Plasma glucose was determined by the enzymatic (hexokinase) method, and plasma insulin by ELISA.

At 6 months, rats from each group were weighed, culled and tissues dissected, weighed and snap-frozen on dry ice (n=7-9 per group).

6.3 Results

6.3.1 Body weights

As described in chapter four, F1 dex animals were significantly lighter than F1 veh animals at birth (Table 4.1). F1 dex males remained lighter at weaning (Figure 6.1). F1 dex animals in both HF and C groups achieved catch-up growth by 7 weeks of age (Figure 6.1).

Analysis of variance revealed that both DHF and VHF rats were significantly heavier than controls of the same prenatal treatment group by 5 weeks of age and thereafter remained heavier throughout the time of follow-up (Figure 6.1).

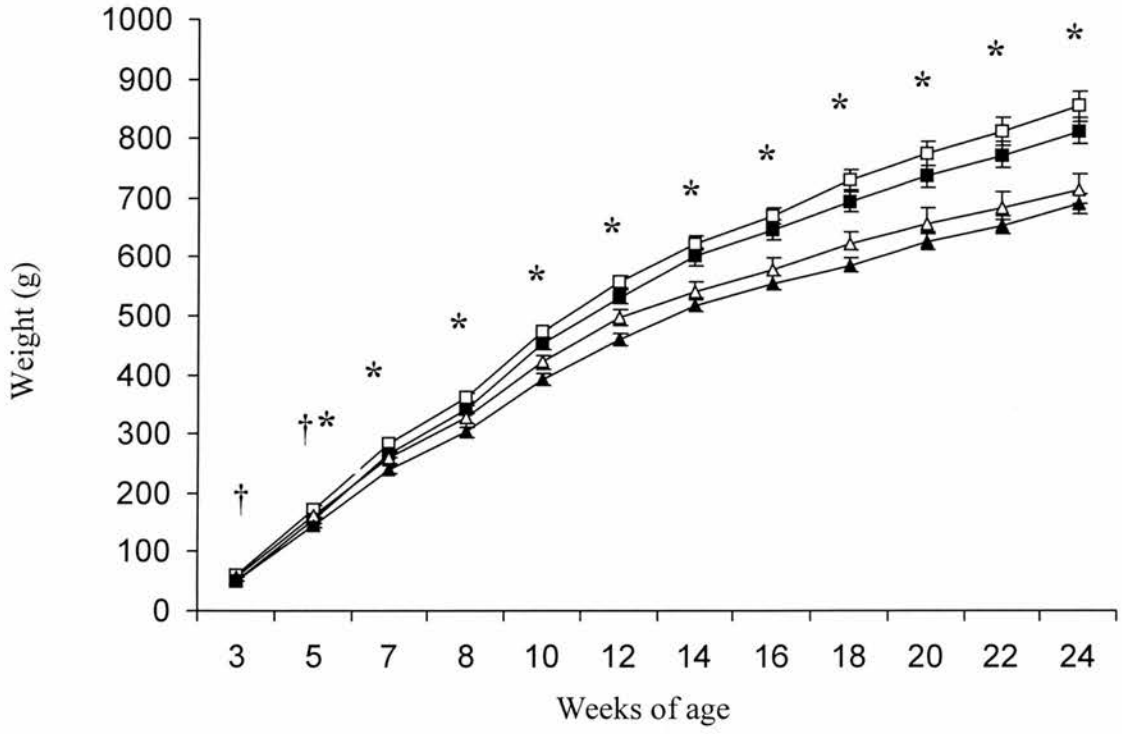


Figure 6.1 Body weights from weaning in HF and C groups.
Data were analysed by ANOVA * denotes $p<0.05$ between HF and C
† denotes $p<0.05$ between dex and veh in both HF and C groups, n=14 VHF, 19 DHF, 14 VC and 15 DC animals.

6.3.2 Glucose tolerance

Animals on the high fat diet were hyperinsulinaemic by 15 weeks of age, although no differences were noted in glucose levels between groups. The high fat diet was associated with higher insulin levels at 30 minutes in both DHF and VHF animals (Figure 6.2).

At 6 months, no differences were noted in glucose levels between groups (Figure 6.3). Against a background of maternal veh, the high fat diet increased insulin levels at 120 minutes (VHF animals), however against a background of maternal dex, the high fat diet increased plasma insulin at 30 and 120 minutes (DHF animals) (Figure 6.3).

6.3.3 Organ weights

At 6 months, both VHF and DHF animals had increased retroperitoneal fat pad weight, and a trend for an increase in hepatic weight (Figure 6.4), however there were no differences between Dex and Veh animals in the same dietary group.

6.3.4 Hepatic triglyceride content [1]

At 6 months both VHF and DHF animals had increased hepatic triglyceride levels (Figure 6.5). Against a background of maternal veh, the high fat diet increased liver triglyceride content, however against a background of maternal dex the high fat diet increased liver triglyceride content to a greater extent, such that hepatic triglyceride content was higher in DHF than VHF animals.

[1] Measurement of hepatic triglyceride content was carried out by Dr Peter Raubenheimer, Endocrinology Unit, University of Edinburgh.

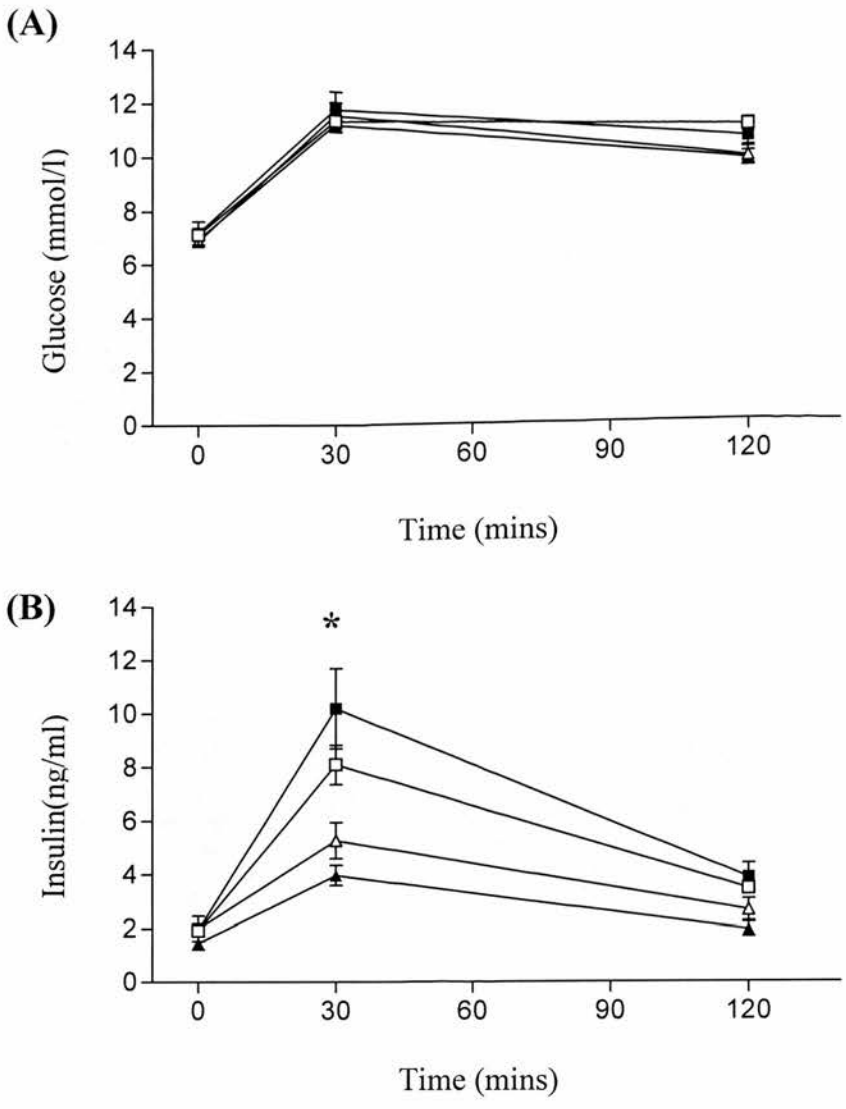


Figure 6.2 Glucose and insulin at 15 weeks.

Glucose (panel A) and insulin (panel B) following a glucose load (n=10 per group). 2-way ANOVA revealed an effect of the high fat diet to increase insulin levels in both dex and veh animals (*$p<0.05$). Post hoc analysis revealed no additional effect of prenatal treatment.

□ VHF
■ DHF
△ VC
▲ DC

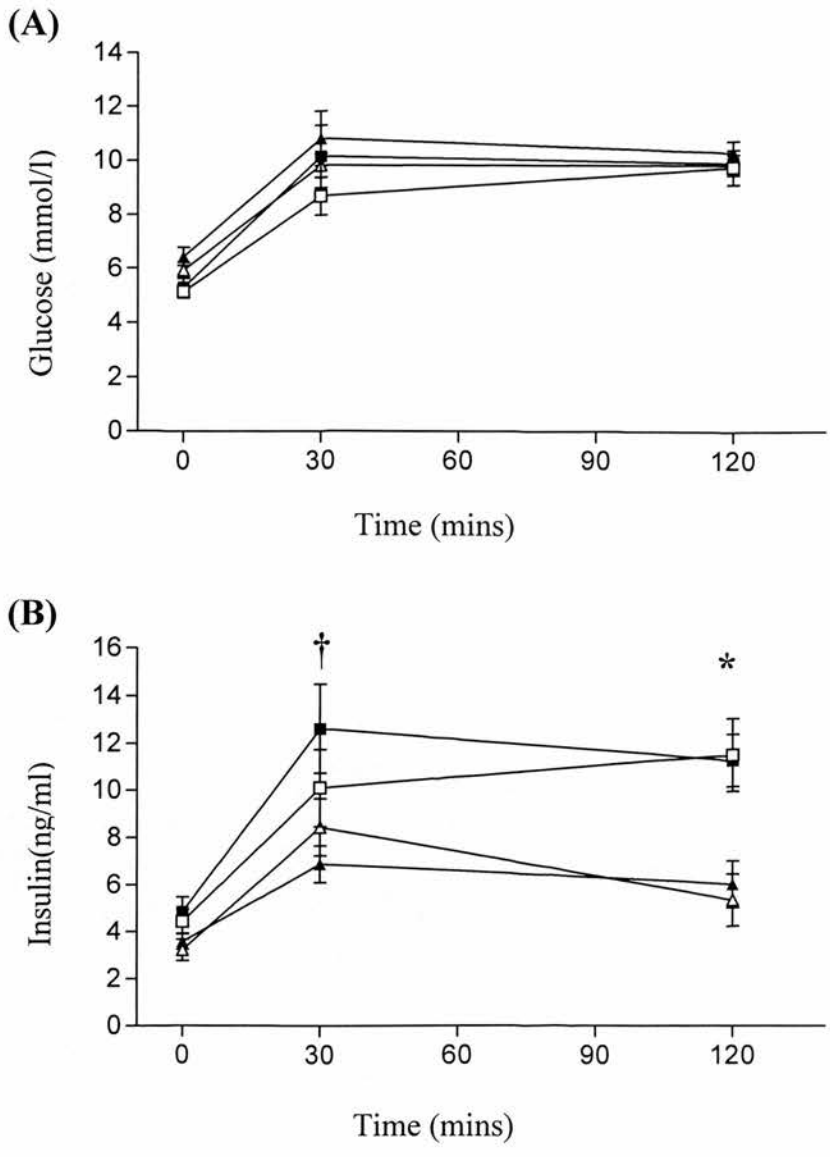


Figure 6.3 Glucose tolerance testing at 6 months.

Glucose (panel A) and insulin (panel B) at 6 months of age (n= 10 VHF, 9 DHF, 9 VC and 8 DC).

2-way ANOVA revealed an effect of the high fat diet to increase insulin levels in both dex and veh animals (*$p<0.05$). Post hoc analysis revealed an additional effect of prenatal dex treatment to increase insulin levels further at 30 minutes in DHF animals (†$p<0.05$).

□ VHF
■ DHF
△ VC
▲ DC

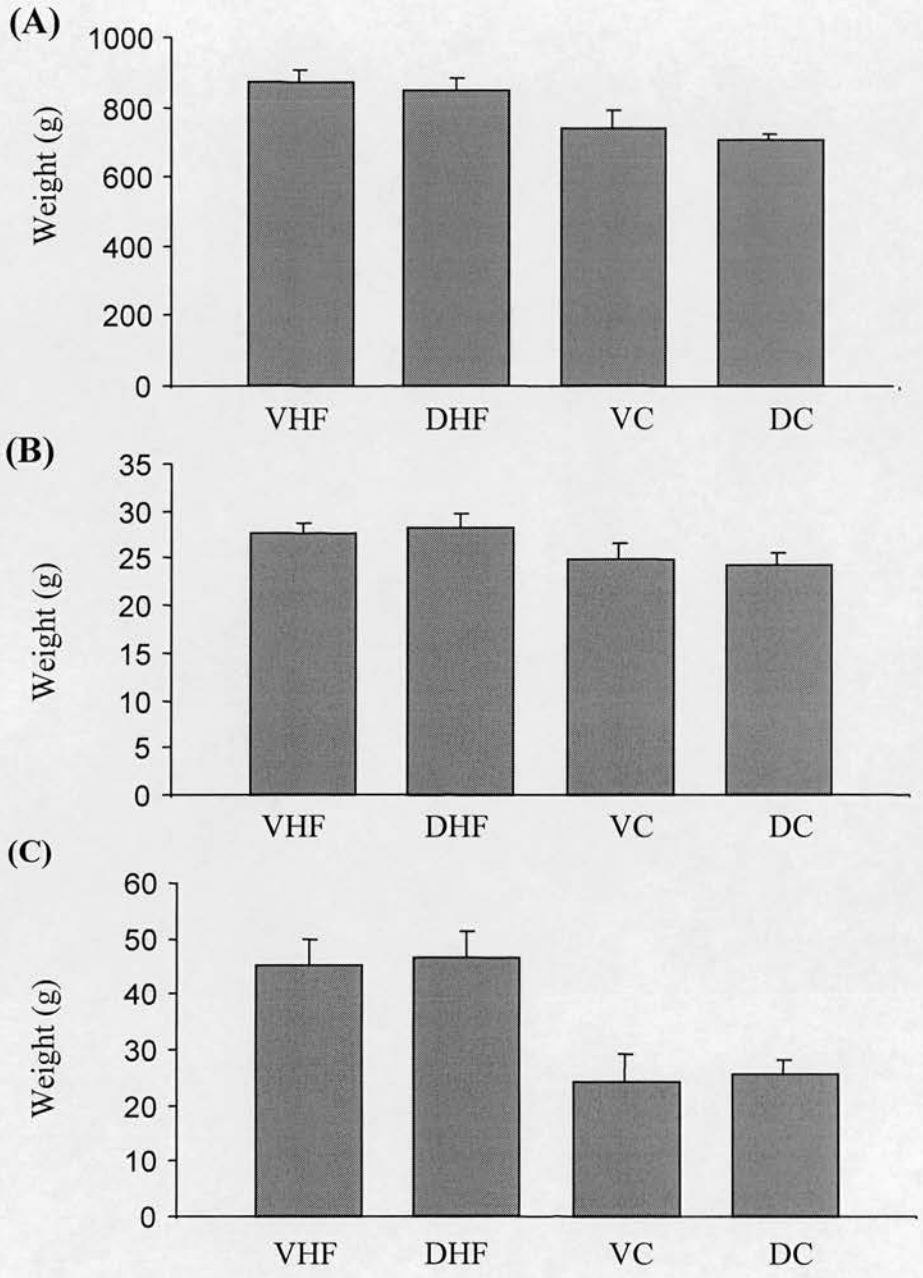


Figure 6.4 Body weight (panel A), liver weight (panel B) and retroperitoneal fat weight (panel C) at 6 months.

Data were analysed by ANOVA. There was an effect of the high fat diet to increase body weight and retroperitoneal fat weight in both dex and veh animals ($p<0.05$, n= 8 VHF, 9 DHF, 7 VC and 8 DC), but no significant effect of prenatal dexamethasone treatment to increase weight further.

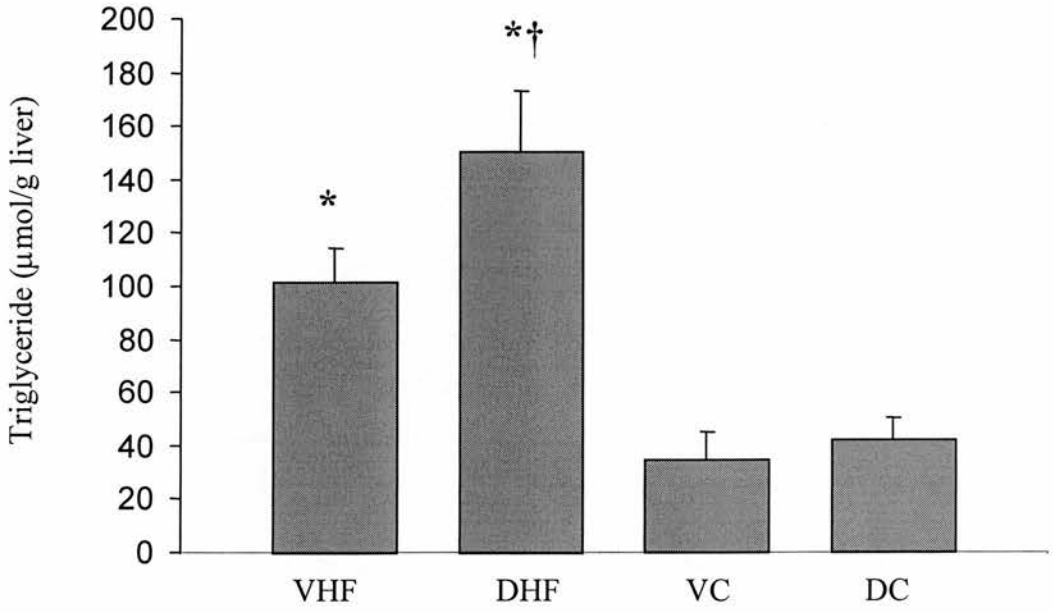


Figure 6.5 Hepatic triglyceride content at 6 months.

Analysis by ANOVA revealed an effect of diet to increase hepatic triglyceride levels in DHF and VHF animals (* $p<0.05$, n= 8 VHF, 8 DHF, 6 VC and 8 DC). Post hoc testing revealed an additional effect of prenatal dexamethasone treatment to further increase hepatic triglyceride content in DHF animals († $p<0.05$).

6.4 Discussion

These results demonstrate that high fat feeding in the dexamethasone-programmed rat is associated with some amplification of the phenotype. In the group of animals, described in this chapter and in chapter four, antenatal dexamethasone treatment was associated with low birth weight, but not with elevated hepatic PEPCK or with glucose intolerance in dex animals on the control diet, consistent with a mild programming phenotype. Postnatal exposure to a high fat diet was associated with obesity and insulin resistance in both dex and veh animals. This effect appeared to be subtly amplified in the dexamethasone programmed group, with hyperinsulinaemia and increased liver fat accumulation, but no increase in obesity, suggesting a specific programming effect on the liver.

In animal models, fetal undernutrition secondary to the use of a low protein or a calorie restricted diet during gestation is associated with reduced birth weight and later metabolic abnormalities (Langley et al 1994, Langley & Jackson 1994, Hales et al 1996, Woodall et al 1996a, Woodall et al 1996b, Garofano et al 1999, Vickers et al 2000, Vickers et al 2001b). In low protein rat models, *in utero* exposure to a low protein diet appears to be associated with insulin resistance and/ or insulin deficiency (Hales et al 1996) and subsequent high fat feeding leads to impaired insulin secretion and impaired glucose tolerance (Holness 1996, Wilson & Hughes 1997). In addition to the metabolic sequelae, maternal calorie restriction in rats is associated with an increased risk of obesity in offspring. Calorie restriction during the first two weeks of gestation was associated with normal offspring birth weight and normal initial postnatal growth, however by 53 days, female offspring had increased fat pad weights although no difference in total body weight compared with controls (Anguita et al 1993). In contrast, male offspring of calorie restricted dams showed lower total body weight and lower fat pad weight by 53 days (Anguita et al 1993). In more recent studies in which calorie restriction throughout gestation was associated with a marked reduction in birth weight and the subsequent development of higher blood pressure and hyperinsulinaemia (Vickers et al 2000, Vickers et al 2001b), malnourished pups of both sexes which were cross-fostered onto control mothers and

weaned onto normal diets did not demonstrate catch-up growth, but did show hyperphagia, more sedentary behaviour and increased fat pad weight relative to body weight (Vickers et al 2000, Vickers et al 2003). Exposure of these offspring to a high fat diet (30% fat) after weaning amplified the metabolic and cardiovascular abnormalities; animals became obese, had higher plasma insulin and leptin levels and higher blood pressure (Vickers et al 2000). The authors suggest that fetal undernutrition induces hyperleptinaemia, hyperinsulinism and insulin and leptin resistance, which may underpin the hyperphagia, obesity and hypertension in this model (Vickers et al 2000). They also speculate that there may be dysregulation of the adipoinsular axis, with impaired leptin suppression of insulin secretion resulting in chronic hyperinsulinaemia, increased adipogenesis and further elevations in plasma leptin (Seufert et al 1999, Vickers et al 2001b).

In the original model of dexamethasone programming in the rat, low birth weight was associated with the subsequent development of glucose intolerance, with fasting and postglucose hyperglycaemia and hyperinsulinaemia (Nyirenda et al 1998). Enhanced hepatic glucose output as a result of programmed hepatic PEPCK overexpression may explain the observed glucose intolerance, indeed as discussed in chapter three, there is no evidence for altered peripheral glucose disposal uptake in this model (Cleasby et al 2003a). In one study, in which dexamethasone-exposed animals had reduced birth weight and postglucose hyperglycaemia, but which did not demonstrate complete catch-up growth, programmed animals did not show any changes in plasma leptin, and had smaller fat pads than controls at 7 months of age (Cleasby et al 2003a).

In the cohort of F1 animals described in this chapter, the programming effect appears to have been very mild; birth weight was reduced in the dex treated animals, but there was no change in hepatic PEPCK. Consistent with this, there was no evidence of glucose intolerance in dex animals on the control diet, at least during a glucose tolerance test. However, there was evidence for a subtle amplification of the programming effect in the dex animals on a high fat diet. Although both dex and veh animals on the high fat diet showed no evidence of hyperglycaemia, the dex animals on a high fat diet showed a greater increase in plasma insulin during the glucose

tolerance test when compared with their littermates on the control diet, than the veh animals, suggesting increased insulin resistance in the DHF group. This is despite no evidence that the dex HF animals were more obese that the veh HF group, as assessed by body weight and fat pad weight.

In agreement with previous reports (Ye et al 2003), the high fat diet induced an increase in hepatic triglyceride content. In human subjects, fatty liver is associated with obesity and diabetes (Marceau et al 1999, Marchesini et al 2001) and is strongly related to insulin resistance (Marchesini et al 2001, Sanyal et al 2001, Pagano et al 2002). The underlying mechanisms are unclear, but may include increased free fatty acid delivery to the liver from visceral fat stores. In obese individuals there may be a finite amount of triglyceride storage in adipose depots, resulting in the diversion and deposition of triglycerides in other tissues including the liver (Garg & Misra 2002). Fatty acids increase hepatic gluconeogenesis, inhibit insulin suppression of glycogenolysis and may cause hepatic insulin resistance (Boden et al 2002, Lam et al 2003). Furthermore, in states of energy excess, fatty acid metabolism may be diverted into esterification, increasing hepatic triglyceride content (Ruderman et al 1999, Lam et al 2003), and further increasing hepatic insulin resistance (Lam et al 2003).

In this study, DHF animals were more hyperinsulinaemic and had greater hepatic triglyceride content than VHF animals, although there was no increase in obesity. This increase in hepatic triglyceride content may be mediated by glucocorticoids. Rats exposed to excess glucocorticoid *in utero* have increased hepatic GR (Nyirenda et al 1998) in the presence of normal or increased circulating corticosterone levels (Levitt et al 1996, Nyirenda et al 1998) and no alteration in hepatic 11β-HSD 1 (Nyirenda et al 1998). The increase in hepatic GR is likely to be associated with an increase in local glucocorticoid action, increasing hepatic insulin resistance. Additionally, programmed animals have increased adipose GR and decreased adipose tissue fatty acid uptake, consistent with insulin resistance in this tissue (Cleasby et al 2003a). Glucocorticoids increase hormone sensitive lipase synthesis;

therefore increased glucocorticoid action and insulin resistance in visceral adipose tissue may increase lipolysis, potentially increasing free fatty acid flux into the liver.

In addition to its role in gluconeogenesis, PEPCK is a rate-limiting enzyme in glyceroneogenesis in both liver and adipose tissue. Glyceroneogenesis, the conversion of pyruvate and amino acids to 3-glycerol phosphate for the synthesis of glyceride-glycerol (Reshef et al 2003), is an abbreviated form of gluconeogenesis and is thought to have a role in the re-esterification of fatty acids in adipose tissue and liver, reducing fatty acid release from adipose tissue and enhancing release from the liver in the form of triglyceride (Reshef et al 1970, Martin-Sanz et al 1990, Reshef et al 2003). However, the potential importance of glyceroneogenesis and the role of PEPCK in the control of fatty acid recycling in adipose tissue and liver in disease remain to be determined. Glucocorticoids stimulate PEPCK gene transcription in liver, but repress it in adipose tissue (Sasaki et al 1984, Meisner et al 1985, Nechushtan et al 1987); adrenalectomy enhances glyceroneogenesis and diminishes fatty acid release from adipose tissue in vitro (Reshef et al 1969), whereas the addition of glucocorticoids to cultured hepatocytes increased the synthesis of triglycerides and stimulated the release of very low density lipoproteins (Martin-Sanz et al 1990). Although in this experiment we did not demonstrate elevated hepatic PEPCK activity in the group of dex animals in which it was measured at weaning; it is an intriguing possibility that in the programmed animal a glucocorticoid-mediated increase in PEPCK expression in the liver might contribute to the increased hepatic triglyceride storage. Conversely, a glucocorticoid-induced downregulation in PEPCK expression in adipose tissue may suppress glyceroneogenesis in fat stores, resulting in an increased flow of free fatty acids to the liver.

Thus, the amplification of the programming effect by obesity may be related to glucocorticoid dependent changes in hepatic metabolism and insulin resistance, which may be altered in the programmed animal. Although time has not permitted further exploration of glucocorticoid signalling within these animals (as described in chapter five), alterations in glucocorticoid metabolism in programmed animals may

underpin the amplification of the programming effect by obesity and require further exploration.

Could this be relevant in the human population? Epidemiological studies demonstrate that low birth weight is a risk factor for the development of obesity during childhood and adulthood (Ong et al 2000), which may modify the risk of later disease (Phillips et al 1994, Lithell et al 1996, Leon et al 1998, Eriksson et al 1999, Forsen et al 2000, Eriksson et al 2001, Law et al 2002) and postnatal nutrition is likely to be important in mediating this (Lucas 1991, Singhal et al 2001, Singhal et al 2003). Low birth weight is associated with later insulin resistance and the metabolic syndrome (Barker 1998), conditions which are associated with fatty liver disease (Marceau et al 1999, Marchesini et al 2001). Thus, in humans, the combination of *in utero* effects and later nutritional status may have important implications for adult health. This animal model may provide important insights into how the postnatal environment may amplify the metabolic and cardiovascular abnormalities associated with low birth weight.

Chapter Seven – Discussion

The aim of this thesis was to study the intergenerational effects of programming by *in utero* exposure to excess glucocorticoid in the rat and to explore the influence of parental *in utero* glucocorticoid exposure on offspring phenotype. We also describe the development of a model of diet-induced obesity, which was subsequently applied to the programming model to examine the amplification of the programming phenotype by obesity. A summary is presented in figure 7.1.

7.1 The intergenerational effects of dexamethasone programming

We have explored intergenerational effects in an animal model of fetal programming, the dexamethasone-programmed rat. In this model, administration of dexamethasone to female Wistar rats during the last week of a three-week pregnancy results in offspring of low birth weight, with elevated hepatic PEPCK activity, which subsequently develop glucose intolerance and hypertension in adulthood (Benediktsson et al 1993, Nyirenda et al 1998). Using this model we have been able to demonstrate a consistent effect in a second (F2) generation (F2 dex and F2 veh). Without any further manipulation, second generation offspring of dexamethasone treated rats are also of low birth weight, with elevated hepatic PEPCK activity. The programming effect appears to be stronger in the F2 generation than in F1 animals, and in male offspring. Male F2 dex offspring are glucose intolerant by four months of age and have higher blood pressures in adulthood. Female F2 dex offspring have some evidence for insulin resistance in adulthood, but do not demonstrate abnormal blood pressure. No intergenerational effects on birth weight, hepatic PEPCK activity, or glucose tolerance were detectable in a third generation.

It has been proposed that the increased expression of PEPCK in this model may be secondary to increased hepatic GR expression in the presence of normal circulating concentrations of corticosterone (Nyirenda et al 1998). We found no differences in circulating corticosterone levels in males or females in any of the generations

studied, but due to time constraints we were unable to explore GR expression. However, given the intergenerational effects we have described on hepatic PEPCK and glucose tolerance, future studies in this model should address GR expression in the second and third generations.

There were a number of sex-specific effects noted in this study. The postnatal growth of males and females in the F1 cohort described in chapter three differed, with males, but not females, achieving catch up growth. However, the intergenerational effects on glucose tolerance and blood pressure in the F2 animals appeared stronger in males. A number of sex-specific effects have been described in animal models of fetal programming (Hales et al 1996, Dean & Matthews 1999, Kind et al 1999, Lingas et al 1999, Dean et al 2001, Lingas & Matthews 2001, Liu et al 2001, Owen & Matthews 2003) and human studies have revealed some sex differences in the long-term disease risk associated with low birth weight (Forsen et al 2000, Walker et al 2002), or exposure to famine prenatally (Ravelli et al 1999). Recent studies have shown different patterns of development of GR and MR in male and female guinea pig brains, which may underlie the sex-specific effects of prenatal glucocorticoid exposure on HPA regulation in this animal (Owen & Matthews 2003). We have not explored in detail the mechanisms of the sex-specific effects in this model, however the differential sensitivity of males and females to programming phenomenon may be due to the influence of sex steroids, sex chromosome effects, sex-specific patterns of development, parental imprinting or differences in maternal care. Indeed, although not apparent in our model, stronger programming effects in females could further amplify a matrilineal pattern of intergenerational inheritance.

In terms of mechanisms, studies demonstrating intergenerational effects of maternal dietary manipulations on birth weight, pancreatic development and glucose homeostasis in rats (Laychock et al 1995, Vadlamudi et al 1995, Martin et al 2000, Srinivasan et al 2003) suggest that *in utero* exposure to an adverse environment might lead to a perpetuation of programming effects in subsequent generations. Antenatal dexamethasone exposure may produce permanent alterations in maternal physiology, which might impact on a developing fetus and lead to a perpetuation of programming effects in subsequent generations. Human studies have suggested that

poor maternal intrauterine growth and a lack of catch up growth may have effects on uterine size, which could have an impact upon fetal growth. In our studies, the F1 dex and veh females described in chapter four showed no difference in body weight at the time of conception, suggesting that maternal size was not a major determinant of offspring size in this model. There are, however, a number of other maternal factors that may result in the intergenerational effects described in this thesis, and which remain to be explored. Although there were no differences in basal corticosterone levels in F1 or F2 females, we did not exclude alterations in peak levels, or changes across the full diurnal rhythm, nor did we measure glucocorticoid levels during pregnancy. Clearly, given the programming effects of glucocorticoid exposure *in utero* in the F1 generation, maternal hypercorticosteronaemia might play a key role in the potentiation of such effects in subsequent generations. Similarly, alterations in maternal insulin levels or blood pressure during pregnancy were not assessed and future studies will address these potential mechanisms in this model.

However, as we have shown, maternal factors are not sufficient to explain all of the intergenerational effects described and both maternal and paternal dexamethasone exposure has an effect on the phenotype of the F2 offspring. Paternal prenatal dexamethasone exposure produced a greater reduction in birth weight in offspring of control mothers than of dexamethasone-exposed mothers, emphasising an important effect of the father on the intergenerational transmission of programming effects. The offspring of dex-programmed fathers also had increased offspring PEPCK expression irrespective of maternal dex exposure, although, unlike birth weight, maternal dex programming appeared a more potent determinant of offspring PEPCK expression. Further phenotyping of the F2 offspring of crosses between dex and vehicle animals including glucose tolerance is awaited. We suggest that these intergenerational effects, which are transmissible from both parents, may be secondary to epigenetic effects on the genome. Indeed, as discussed in chapter four, recent studies have suggested that environmental influences such as glucocorticoids may have epigenetic effects on the offspring, and that some epigenetic modifications may be transmitted from either parent, or both.

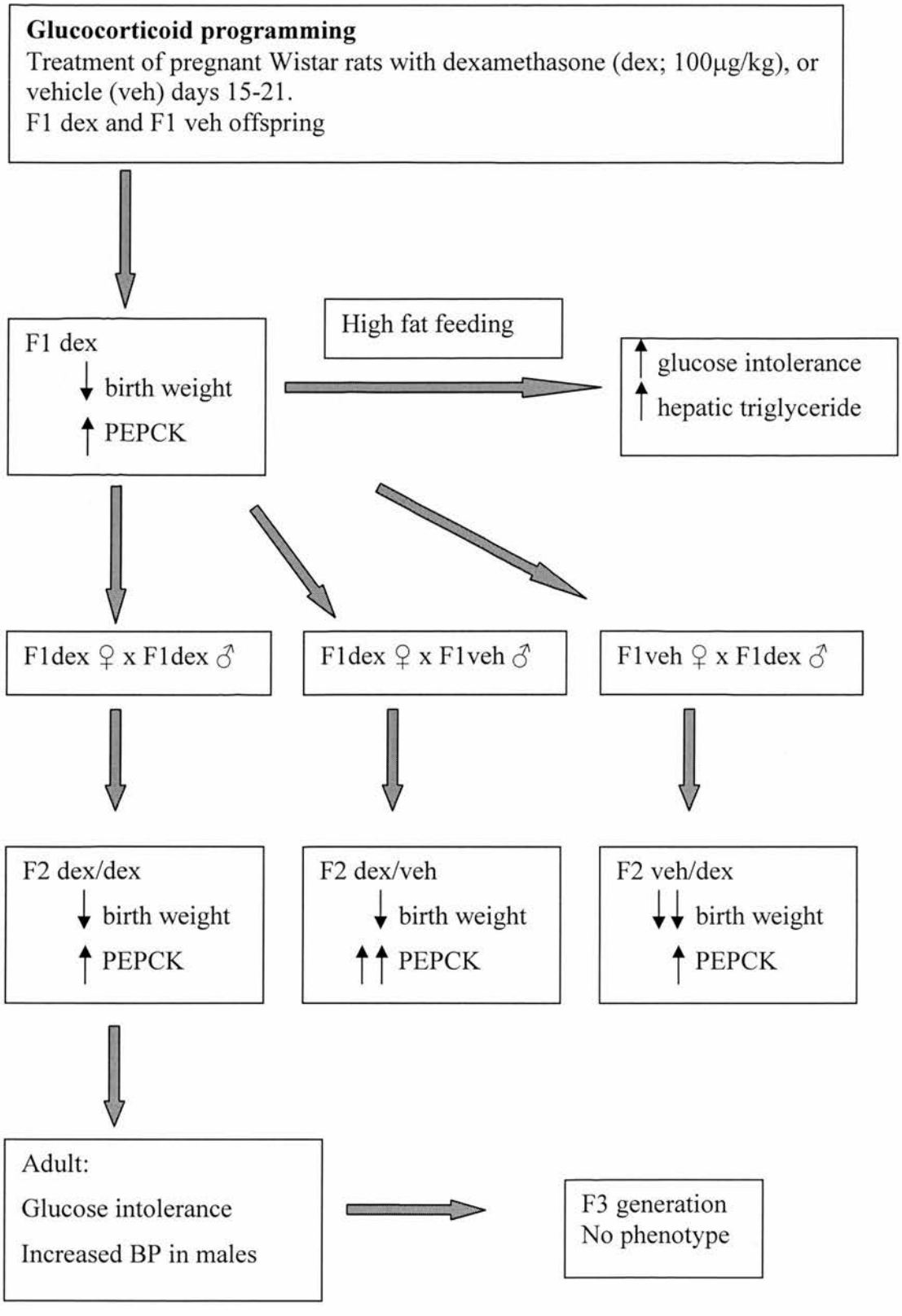


Figure 7.1 Summary of results

7.2 The amplification of programming by obesity

In chapter four we describe the development of a model of diet-induced obesity in male Wistar rats, in which we examined changes in glucocorticoid metabolism. Exposure of Wistar rats to a high fat diet is not associated with the same changes in glucocorticoid signalling and metabolism that have been described in other animal models of obesity such as the obese Zucker rat, in which reactivation of corticosterone by 11β-HSD 1 is impaired in liver, but enhanced in omental adipose tissue. Similar alterations in glucocorticoid metabolism by 11β-HSD 1 have been demonstrated in human obesity. In this study we have shown that in the Wistar rat, short-term exposure to a high fat diet induces alterations in glucocorticoid metabolism, with decreased 11β-HSD 1 activity in liver and fat depots, and increased hepatic 5β-reductase activity, in the absence of obesity, hyperinsulinaemia, hypercorticosteronaemia or changes in hepatic or omental adipose GR. These alterations are not however, maintained in the longer term, despite the development of obesity and insulin resistance. We suggest that the early down regulation of 11β-HSD 1 in this model, together with the upregulation of hepatic 5β-reductase activity results in increased glucocorticoid clearance and may reflect a protective mechanism against the metabolic consequences of a high fat diet. This hypothesis is supported by studies demonstrating that mice with a targeted disruption of the 11β-HSD 1 gene are protected from some of the metabolic consequences of obesity (Kotelevtsev et al 1997). The mechanisms underlying this remain to be determined, however these data are in agreement with previous studies that have demonstrated that alterations in 11β-HSD 1 and GR do not appear to be dependent on hyperinsulinaemia or hypercorticosteronaemia (Livingstone et al 2000b). It is possible that very long term high fat feeding in this model might be associated with further alterations in 11β-HSD 1 and GR; a further study is already in progress to examine the effects of very long term (40 weeks) high fat feeding in Wistar rats on glucocorticoid metabolism.

In order to explore the effects of obesity in this programming model, we applied this model of dietary obesity to F1 dex and F1 veh males. In both F1 dex and F1 veh males, the high fat diet induced weight gain, increased retroperitoneal fat pad weight

and caused hyperinsulinaemia and increased liver triglyceride content. However, by 6 months of age there were no differences between dex and veh animals in weight gain or retroperitoneal fat pad weight. Thus, antenatal dexamethasone exposure does not appear to confer increased sensitivity to obesity induced by high fat feeding. However, F1 dex animals did show greater hyperinsulinaemia and increased hepatic triglyceride accumulation in response to the high fat diet. Again, the mechanisms underlying this remain to be determined, but the increased hepatic lipid accumulation may reflect decreased hepatic insulin sensitivity. Further studies are required to determine whether alterations in glucocorticoid signalling are important in the pathogenesis of hepatic triglyceride accumulation in programmed animals.

7.3 Implications for human populations

Epidemiological studies have demonstrated that there is an important relationship between early life experience and subsequent adult disease, and additionally, results from human and animal studies suggest that 'programmed' effects may be transmitted through subsequent generations. The thrifty phenotype hypothesis includes the concept that 'the poorly nourished mother essentially gives the fetus a forecast of the nutritional environment into which it will be born' (Hales & Barker 2001). Physiological adaptations prepare the fetus for the same extrauterine conditions, optimising survival at least until after reproduction, under conditions of stress or deprivation. Such non-genomic influences on fetal growth and development acting across a number of generations would help to ensure continued population survival. However, these intergenerational effects of fetal programming would only be advantageous to population survival when environmental conditions remain consistent over several generations. Any rapid change in the environment puts the 'programmed' offspring at risk of hypertension and glucose intolerance. This is clearly seen in developing countries where peoples leading 'traditional' lifestyles have a low prevalence of type 2 diabetes (King & Rewers 1993). The prevalence of diabetes, hypertension and cardiovascular disease in such populations increases rapidly with urbanisation or migration to other countries, associated with changes in

diet, exercise and the resultant increase in obesity (Cruickshank et al 2001, Fall 2001).

Transgenerational programming effects may therefore lead to the inheritance of a predisposition for low birth weight and cardiovascular risk and have important consequences for cardiovascular risk across a number of generations for populations in both developed and developing societies. However, attempts to investigate the underlying mechanisms of intergenerational effects on birth weight and cardiovascular risk factors using epidemiological studies in human populations are limited by a number of factors, including genetic differences between populations, postnatal environment, lifestyle and diet. Animal models of intergenerational programming in which environmental and genetic variations are minimised are therefore valuable tools in which to dissect and validate potential mechanisms. This model of intergenerational programming provides an exciting opportunity to explore the role of programmed maternal factors and epigenetic influences in the intergenerational transmission of programming effects on birth weight and later disease risk.

Obesity is linked with a number of health consequences including type 2 diabetes and cardiovascular disease and additionally, alterations in glucocorticoid secretion and metabolism have been shown in human obesity and in animal models. In the model of fetal programming used in these experiments, alterations in glucocorticoid signalling appear to be important mediators of the long-term consequences of programming. We have shown that programmed animals appear to be more susceptible to the metabolic consequences of a high fat diet, in the absence of increased obesity, which may be related to glucocorticoid-dependent changes in hepatic metabolism and insulin resistance. These results suggest that the combination of obesity and adverse antenatal environment may interact to alter hepatic insulin sensitivity in this model.

Epidemiological studies in humans suggest that obesity is an important modifier of the risks associated with low birth weight and thus, in humans as well as animal

models, the combination of in utero effects and later nutritional status may have important implications for adult health. This animal model will allow us to further explore how obesity may interact with programming effects to amplify the metabolic and cardiovascular abnormalities associated with low birth weight.

To conclude, epidemiological studies and animal models suggest that intergenerational effects of fetal programming could be associated with major public health implications for populations in both developed and developing worlds. There may be unforeseen long-term and intergenerational effects of interventions that impact on early human development. Indeed, we have shown that antenatal glucocorticoid exposure is associated with intergenerational effects on birth weight and on cardiovascular risk factors in this animal model. Furthermore, postnatal environmental factors such as diet may modify the risk of disease associated with low birth weight. The animal models developed in this thesis will allow us to explore further the mechanisms of intergenerational programming effects and the consequences of postnatal nutrition and obesity.

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